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IDENTIFICATION OF AN INTEGRAL MEMBRANE PHOSPHOLIPASE IN HUMAN LIVER UTILIZING ALKALINE PHOSPHATASE AS A SUBSTRATE

by

BRADLEY ALLAN HAMILTON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

SPRING, 1991

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled IDENTIFICATION OF AN INTEGRAL MEMBRANE PHOSPHOLIPASE IN HUMAN LIVER UTILIZING ALKALINE PHOSPHATASE AS A SUBSTRATE submitted by BRADLEY ALLAN HAMILTON in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL PATHOLOGY.

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L.E. McGann

Date: Nov. 30, 1990

To Allyson

and my mother and father

ABSTRACT

Plasma membranes from human liver contain an enzyme activity, alkaline phosphatase converting activity (ALPCA), that is capable of converting hydrophobic alkaline phosphatase to a hydrophilic form through removal of its phosphatidylinositol membrane anchor. This formed the basis of an assay for the enzyme in that hydrophobic alkaline phosphatase adsorbs to octyl-Sepharose CL-4B, whereas the hydrophilic form does not. Treatment of liver plasma membranes with butanol, pentanol, or Triton X-100 disrupts the membrane to allow endogenous ALPCA to convert hydrophobic alkaline phosphatase to hydrophilic dimers. This ALPCA is solubilized from the plasma membrane with non-ionic detergents with low critical micelle concentrations such as Triton X-100, is unaffected by protease inhibitors, and converts both purified hydrophobic liver and placental alkaline phosphatases. However, it shows no activity towards membrane-bound forms of alkaline phosphatase, phosphatidylinositol, or phosphatidylcholine. This enzyme also cleaves the glycosyl-phosphatidylinositol anchor from variant surface glycoprotein. These data indicate that the converting activity is a phospholipase with specificity for

glycosyl-phosphatidylinositol. Analysis of the lipid hydrolysis products of variant surface glycoprotein by thin layer chromatography and the protein hydrolysis products of placental alkaline phosphatase by polyacrylamide gel electrophoresis following treatment with ALPCA indicate that the enzyme has a phospholipase D specificity.

The enzyme has an acidic pH optimum, is relatively heat stable, and is stimulated by hydrophobic alcohols, such as butanol and pentanol. ALPCA was inhibited by 1,10-phenanthroline and EGTA but activity could be recovered by zinc chloride and calcium chloride, respectively, suggesting the involvement of these ions in the enzyme's activity. Mercuric sulfate inhibition suggests the involvement of a thiol group at the active site. HPLC gel filtration chromatography of ALPCA determined a M_r of 120 000. ALPCA is present in approximately the same amount in liver plasma membranes and whole liver whereas serum contains approximately 10x this amount.

In conclusion, the pH and inhibition profiles, substrate specificity, bond specificity, and M_r suggest an identity between the glycosyl-phosphatidylinositol phospholipase D in liver plasma membranes and the phospholipase D in mammalian plasma.

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LIST OF ABBREVIATIONS

ALPCA	alkaline phosphatase converting activity
ALP	alkaline phosphatase
VSG	variant surface glycoprotein
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
SDS	sodium dodecyl sulfate
NP-40	Nonidet P-40
M_r	relative molecular weight
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
PMSF	phenylmethylsulfonyl fluoride
PI	phosphatidylinositol
PI-PLC	phosphatidylinositol phospholipase C
G-PI	glycosyl-phosphatidylinositol
G-PI-PL (D)(C)	glycosyl-phosphatidylinositol phospholipase (D) (C)
AMP	2-amino-2-methyl-1-propanol

CHAPTER ONE

ALKALINE PHOSPHATASE

A. HISTORICAL

The concept of phosphatases as a separate group of enzymes was suggested in 1907 although the origin of the word, phosphatase, did not occur until 1916 (1). Acid and alkaline were proposed to distinguish between phosphatases with pH optima around 5 and those around 9, respectively (2). Alkaline phosphatase (ALP) was formally adopted as the trivial name for this enzyme (orthophosphoric-monoester phosphohydrolase) in 1961 and assigned the enzyme commission number 3.1.3.1. (3).

The earliest technique used to assay the phosphohydrolysis catalyzed by ALP was to measure the orthophosphate released. The synthetic phosphoester, p-nitrophenylphosphate, used today, has greatly increased the convenience and sensitivity of ALP assays.

ALP is widely distributed in nature, occurring in organisms such as algae, slime mold, bacteria, invertebrates, and vertebrates (3). In vertebrates, ALP is localized to the plasma membranes of cells and is found in large amounts in the small intestine, kidney, developing skeleton, and human placenta at term (4, 5, 6, 7).

B. ISOENZYMES OF ALKALINE PHOSPHATASE

It is now known that there are at least four gene loci for ALP (8). The gene encoding the liver/bone/kidney or tissue non-specific ALP has been localized to near the end of the short arm of chromosome 1(p36.1-p34) whereas the genes encoding for placental, germ cell, and intestinal ALPs are closely linked near the end of the long arm of chromosome 2(q34-q37) (9, 10). Evidence summarized below, led to the postulate of an ancestral gene that gave rise to two branches: the liver/bone/kidney gene and the intestinal, placental, and germ cell genes (Fig. I-1 adapted) (11).

Thermostability, tissue specific inhibitors, and immunological methods have proven useful in discriminating between the different alkaline phosphatases (ALPs) (8). Placental and germ cell ALPs can be heated at 65°C for 1 h without loss of activity whereas intestinal and tissue non-specific ALPs are both inactivated by this treatment (12). Intestinal ALP is slightly more thermostable than the liver/bone/kidney ALPs and ALP from liver is slightly more thermostable than the enzyme from bone (13, 14).

Placental, germ cell, and intestinal ALPs are much more sensitive to inhibition with L-phenylalanine than the liver/bone/kidney ALPs which are much more sensitive

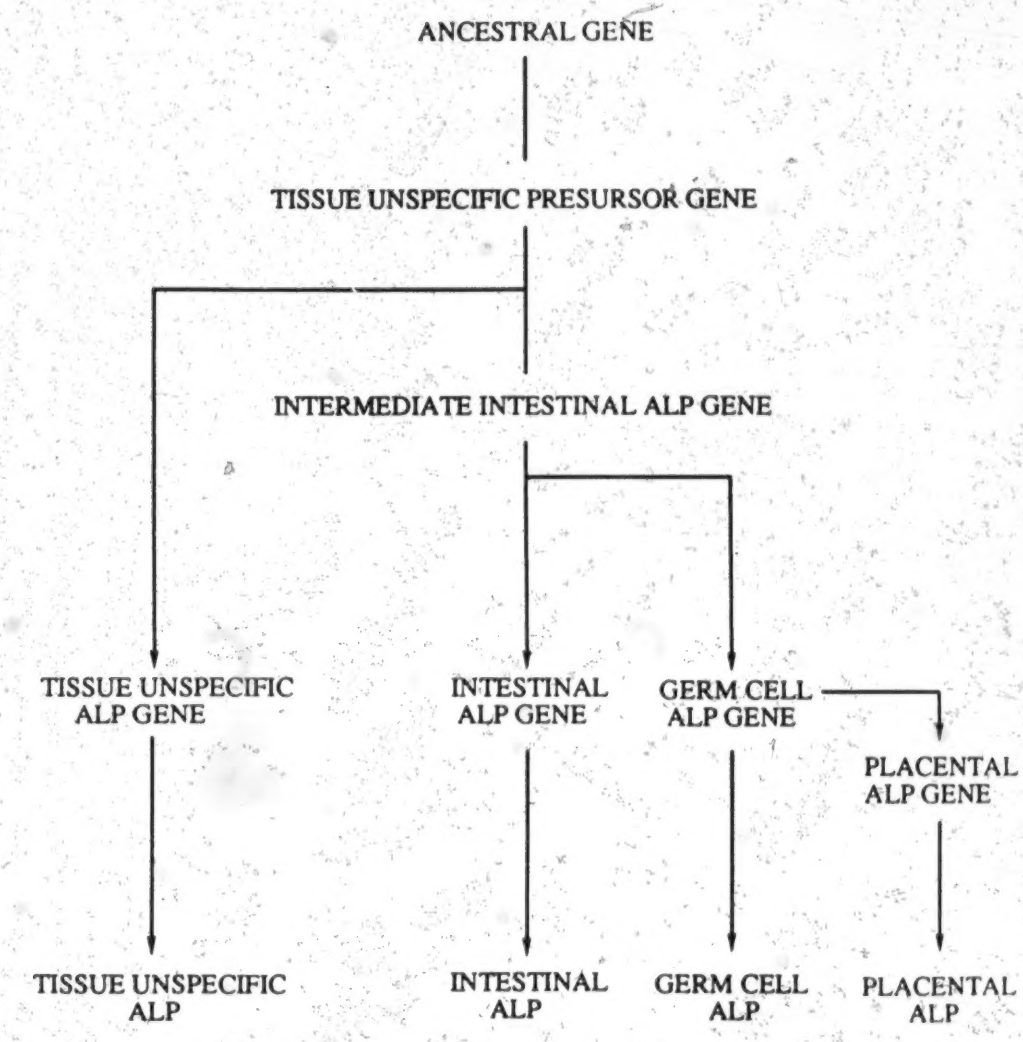

FIGURE I-1 Ancestral development of Alkaline phosphatase

to L-homoarginine inhibiton than the other three ALPs (15). L-Phenylalanylglycylglycine inhibits placental, intestinal,and germ cell ALPs at a concentration 30 times less than that required for inhibition of the liver/bone/kidney ALPs, and also differentiates between placental and germ cell ALPs. Germ cell ALP is much more sensitive to L-leucine inhibition than the other forms whereas the liver/bone/kidney ALPs are much more sensitive to levamisole inhibition than the other isoenzymes (16, 17).

Antibodies to the various isoenzymes have provided a method of differentiation. Antibodies raised to purified placental ALP cross-react with germ cell and intestinal ALP but not with the liver/bone/kidney ALPs (18,19). Antibodies raised to intestinal ALP cross-react with placental and germ cell ALPs but not with the liver/bone/kidney ALPs. Antibodies raised to the liver/bone/kidney ALPs do not cross-react with the other isoenzymes. This suggests that some of the antigenic determinants found on placental ALP are also found on germ cell and intestinal ALPs (8). Amino acid sequences of the various ALPs reveal 98% homology between placental and germ cell ALP, 87% homology between placental and intestinal ALP and approximately 50% homology between placental and liver/bone/kidney ALPs or intestinal and liver/bone/kidney ALPs (8, 20, 21).

C. STRUCTURE OF ALKALINE PHOSPHATASE

ALP belongs to a group of membrane proteins which are anchored to cell membranes by covalent attachment of a glycosyl-phosphatidylinositol (G-PI) structure to the carboxyl-terminus (Chapter 2, Section B). Under normal conditions, identical monomers dimerize and interact with zinc to form the active enzyme in Escherichia coli and in mammals, although tetramer-like ALP has been observed in E. coli and in human liver plasma membranes (3, 22, 23, 24). Butanol and non-ionic detergents can solubilize a tetramer-like ALP from human liver plasma membranes, which can then be converted to a dimer-like form by bacterial phosphatidylinositol phospholipase C (PI-PLC), suggesting that the forces holding the two pairs of dimers together resides in the phosphatidylinositol (PI) anchor (25). This PI anchor is also responsible for the ALP protein being hydrophobic as assessed by adsorption to octyl-Sepharose and Triton X-114 phase partitioning, while removal of the PI anchor results in hydrophilic ALP (26, 27).

Each dimer of ALP requires 2 zinc atoms for catalytic activity, but 4 must be present for maximum activity (29). One pair of zinc atoms is associated with enzymatic activity and can be easily removed, while the other pair is associated with the structure of the

molecule. Magnesium ions also enhance ALP activity but are not required for activity (30).

D. MECHANISM OF CATALYSIS

ALP is non-specific and can hydrolyze many phosphorous compounds regardless of the chemical nature of the leaving group (31). The reaction mechanism for ALP is shown in Figure I-2. ALP noncovalently binds the substrate, cleaves the phosphomonoester bond, and becomes phosphorylated at a serine residue at the active site. This covalent phosphorylenzyme is then converted to a noncovalent complex from which orthophosphate is released to regenerate the free enzyme. As well as hydrolysis of phosphomonoesters, ALP is involved in hydrolysis of phosphoproteins and in phosphate transfer (3). Intestinal ALP is capable of dephosphorylating casein and purified liver ALP is capable of dephosphorylating phosphohistones and membrane phosphoproteins, with a selectivity for phosphotyrosine residues in proteins (32, 33, 34). The phosphotransferase activity of ALP occurs if an alcohol accepts the phosphoryl group from the phosphorylenzyme intermediate (Fig. I-2) (35).

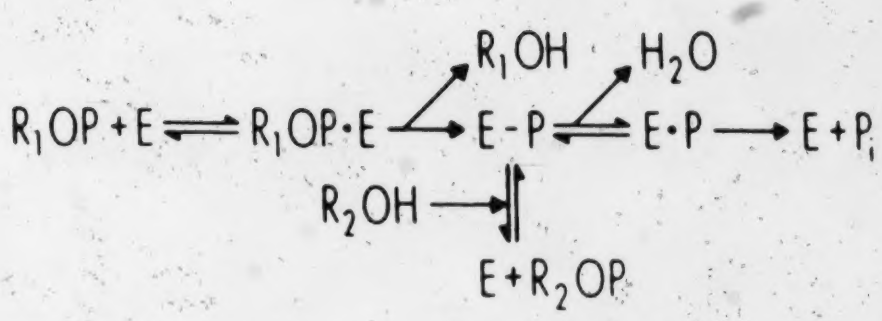

FIGURE I-2 Mechanism of Catalysis of Alkaline Phosphatase. E represents an ALP subunit, E-P the covalent phosphorylenzyme, E.P the noncovalent enzyme substrate complex, P_i inorganic phosphate, R_1OP a phosphate-containing molecule, R_2OH a hydroxyl-containing molecule.

E. CLINICAL APPLICATIONS

ALP measurements are widely used in the diagnosis of diseases involving the skeleton, the hepatobiliary system, and the placenta. An increase in osteoblastic activity in bone results in an increase in circulating ALP, and Paget's disease of the bone has resulted in some of the highest serum ALP values on record with values 30x normal being recorded (36). Osteomalacia and rickets are also associated with increased osteoblast activity and hence have ALP levels that are 3-4x normal (37).

Liver or biliary tract problems which impair bile flow will cause an increase in serum ALP, explained by a stimulation of ALP synthesis in the liver (37). Bile acids may also act as a detergent to release the ALP into the bile and then the serum (38).

Placental ALP is responsible for the increased serum ALP observed in the last third of normal pregnancy (39). The reappearance of placental ALP or germ cell ALP can occur in some maliganant diseases (40).

Hypophosphatasia is an autosomal recessive disease characterized by defective bone mineralization and by a generalized decrease in tissue-nonspecific ALP (41, 42). This is believed to be due to the failure of the liver and bone to contribute normal levels of ALP to the circulation. Infantile type hypophosphatasia is often

fatal, whereas adults suffer from recurrent bone fractures. Phosphoethanolamine, inorganic phosphate and pyridoxal-5'-phosphate accumulate in the urine in this disease suggesting that they are natural substrates for tissue-nonspecific ALP. Defective bone mineralization and tooth formation suggests that ALP is necessary for proper bone and tooth formation.

There are variations in serum ALP with age. Newborns have slightly higher levels than adults and it continues to rise to 2.5-3x adult levels in the first year (37). ALP declines to 1.5-2.5x adult levels by the end of the second year and remains somewhat constant at this elevated level through childhood and early adolescence. A slight increase in ALP may occur in adolescence followed by a steady decline to adult levels in the late teenage years.

F. PHYSIOLOGICAL FUNCTIONS

Despite 60 years of research and thousands of publications the physiological function of ALP is not understood. Since its discovery, ALP was believed to function as a phosphohydrolase. (1). Phosphohydrolases at the cell surfaces, such as ALP, could hydrolyze nonpermeable phosphoesters to orthophosphate and organic residues, which could then be transported into the cell (43).

The location of ALP on cell surfaces has led to the belief that it is involved in cell aggregation and the transport of specific substances. The aggregation of cells in slime mold is associated with an increase in ALP, whereas a lack of aggregation is associated with decreased ALP (44). Phosphate transport, thiamine transport, and fat absorption occur under conditions at which ALP is active and in areas where the concentration of ALP is high (11, 45, 46). Due to the relationship between decreased ALP and hypophosphatasia, ALP has been implicated in bone formation. A close relationship exists between ALP positive cartilage and calcification, and transformation of fibroblasts into osteogenic tissue was preceded by a local rise in ALP activity (47). The ability of ALP from liver plasma membranes to dephosphorylate plasma membrane phosphoproteins suggest that it may be involved in controlling some aspects of plasma membrane function (34).

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CHAPTER TWO

PHOSPHATIDYLINOSITOL ANCHORS AND PHOSPHOLIPASES

A. PHOSPHATIDYLINOSITOL ANCHORED PROTEINS

The mode of anchorage for membrane proteins can vary markedly. Proteins can be almost completely embedded in the lipid bilayer or contain stretches of hydrophobic amino acids which enter the lipid bilayer to anchor the protein (1). Fatty acids, such as myristic and palmitic acid, covalently attached to protein have recently been found to be responsible for anchoring membrane proteins (2). A new and ever increasing group of functionally diverse membrane proteins, which are anchored by a covalently attached glycosyl-phosphatidylinositol (G-PI) moiety, has been found in many different organisms (Table II-1) (3, 4). The initial evidence for proteins being anchored by G-PI was their susceptibility to release by bacterial phosphatidylinositol phospholipase C (PI-PLC) enzymes. Chemical analysis of proteins anchored by G-PI has confirmed the existence of a G-PI moiety although there is evidence of G-PI anchored proteins which are resistant to release by bacterial PI-PLCs. In the case of acetylcholinesterase of human erythrocytes, this resistance is due to an extra palmitic acid attached to the inositol and inserted in the membrane (5). Removal of the palmitic acid allows release by PI-PLC.

TABLE II-1 REPRESENTATIVE LIST OF PROTEINS ANCHORED BY GLYCOSYL-PHOSPHATIDYLINOSITOL

Protein	Source
Alkaline phosphatase	Mammalian tissues (6)
5'-Nucleotidase	Mammalian tissues (7)
Acetylcholinesterase	Mammalian blood cells, Torpedo electric organ (8,9)
Trehalase	Mammalian tissues (10)
Decay accelerating factor	Human blood cells (11)
Variant surface glycoprotein	Trypanosoma brucei (12)
Dipeptidase	Pig kidney, sheep lung (13,14)
Thy-1	Mammalian brain and lymphocytes (15,16)
Thy-3	Mouse lymphocytes (17)
Carcinoembryonic antigen	Human tumor cells (18)
CD14	Human monocytes (19)
gp63 Protease	Leishmania major (20)
p76 Protease	Plasmodium falciporum (21)
Alkaline phosphodiesterase I	Rat tissues (22)
Aminopeptidase P	Pig and human kidney (23)
Heparan sulphate proteoglycan	Rat hepatocytes (24)

B. STRUCTURE AND PROCESSING OF THE GLYCOSYL-PHOSPHATIDYLINOSITOL MOIETY

G-PI structures have been studied in several proteins but most extensively in variant surface glycoprotein (VSG) from Trypanasoma brucei. Common features of a G-PI anchor are shown in Fig. II-1 (adapted, '1). Ethanolamine has been shown to be linked via an amide bond to the α-carboxyl group of a variable C-terminal amino acid (4, 5). This residue is cys in Thy-1, asp in placental ALP, and asp and ser in two forms of VSG (1, 25, 26). G-PI proteins other than VSG contain an additional 1 to 2 ethanolamine residues. In VSG of Trypanasoma brucei, the hydroxyl group of the ethanolamine residue is linked to the 6-position of a mannose residue via a phosphodiester bond (4). The number of mannose, galactose, and galactosamine residues in the glycan varies greatly among different proteins (1). Glucosamine has been found to be linked to myo-inositol at the 6 position in VSG via a glycosidic linkage (4).

Fatty acids found on G-PI proteins vary from organism to organism with higher eukaryotes exhibiting a variety of saturated and unsaturated fatty acids, while Trypanasoma brucei shows exclusively myristic acid (4). Human placental ALP was found to contain 2 moles of ethanolamine and 1 mole each of palmitate, stearate,

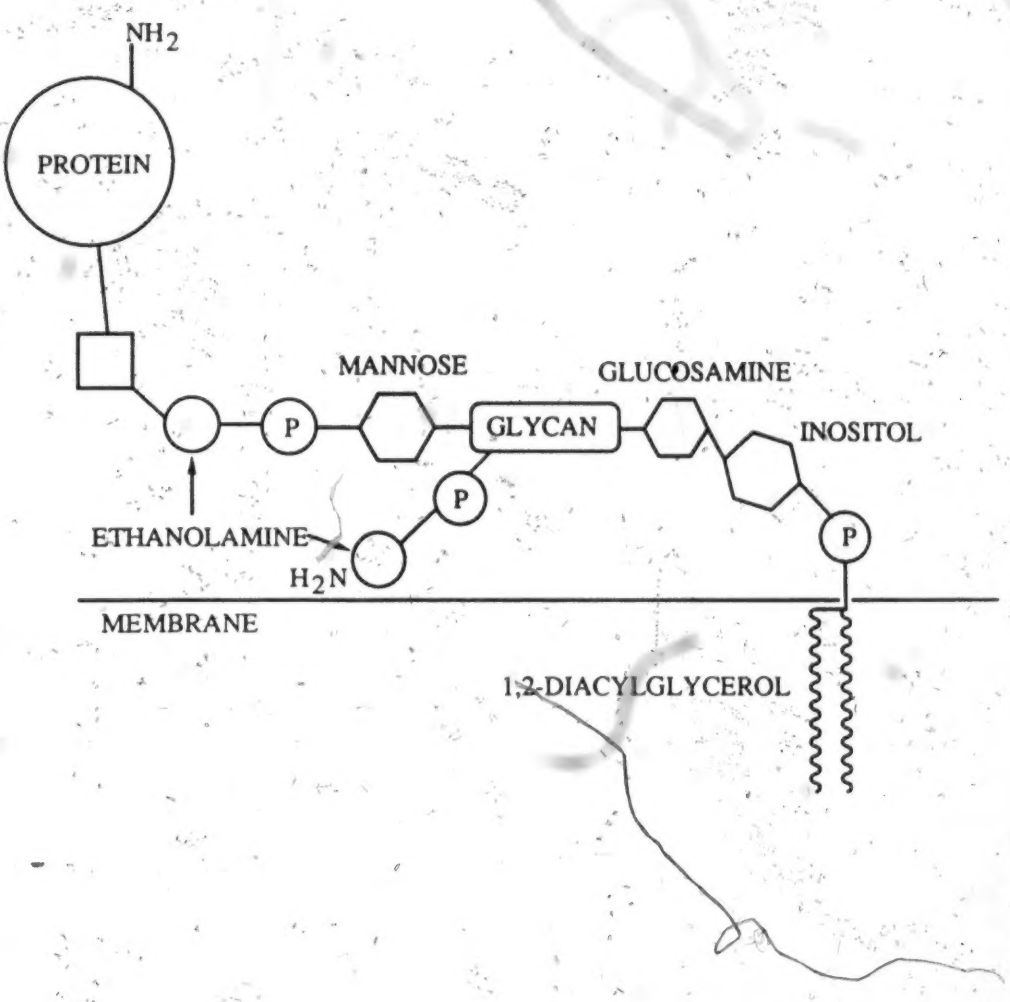

FIGURE II-1 General Scheme of a Glycosyl-phosphatidylinositol anchor

glycerol, and inositol per subunit (27).

The conservation of structure seen in the anchors of different G-PI proteins suggest that they may follow the same biosynthetic pathway. A proposed model for the attachment of the G-PI anchor to placental alkaline phosphatase is shown in Fig. II-2 (adapted) (28). cDNA sequences for G-PI anchored proteins encode an N-terminal signal peptide that directs the protein to the endoplasmic reticulum and a sequence of 20-30 hydrophobic amino acids at the C-terminus (29). The sequence of this C-terminal signal peptide, necessary for the attachment of the PI anchor, was investigated for placental ALP (26). It was found that an uncharged, predominantly hydrophobic amino acid sequence of a minimal length must be present for PI anchoring to occur. Placental ALP with 13 or fewer hydrophobic amino acids at the C-terminus resulted in hydrophilic proteins that were secreted, whereas placental ALP with 17 hydrophobic amino acids at the C-terminus, regardless of the sequence, resulted in PI anchoring. A similar phenomenon was observed for decay accelerating factor except that in addition to the stretch of hydrophobic amino acids, it was found that a suitable cleavage and attachment site is necessary for phosphatidylinositol anchorage (30). Removal of amino acids adjacent to the C-terminal signal peptide resulted in the protein being secreted, not PI anchored. Known

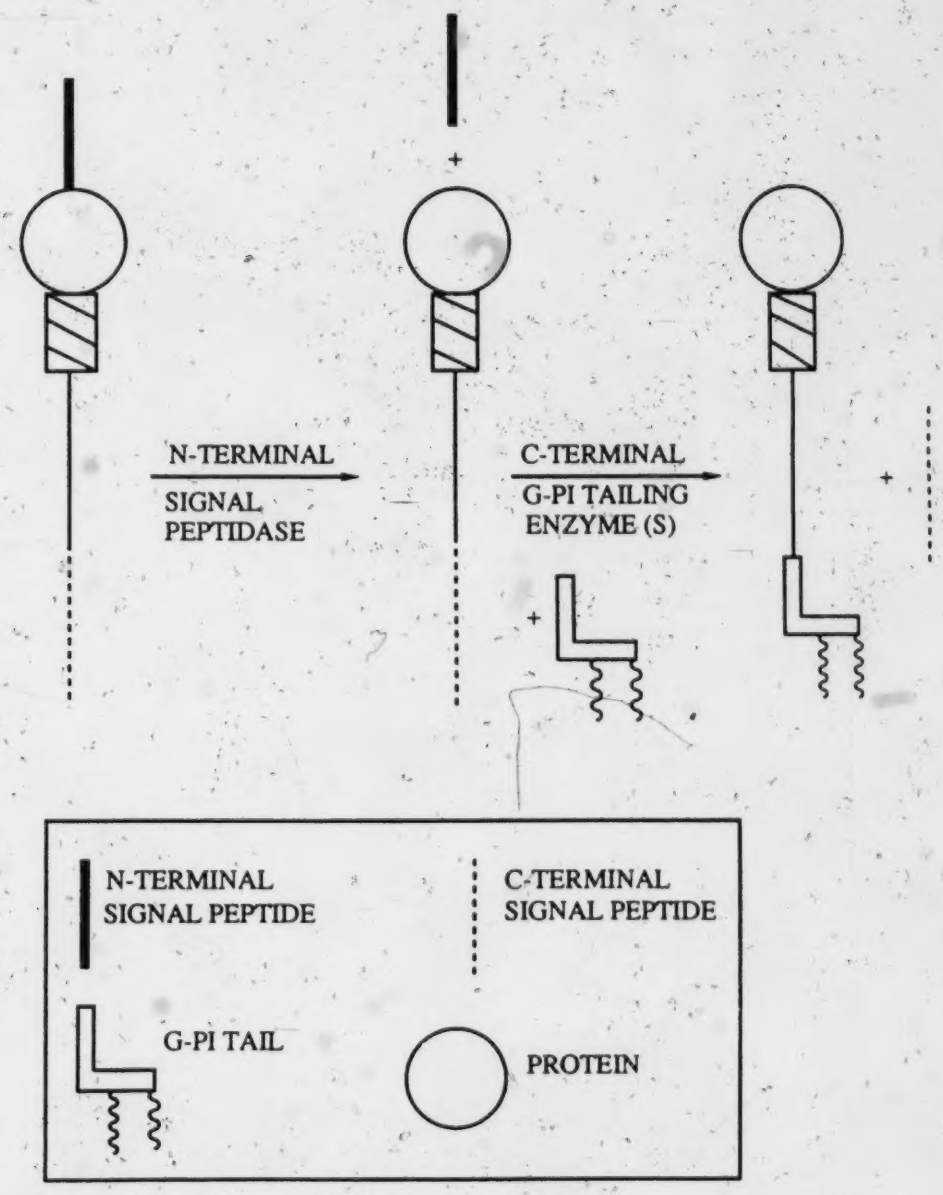

FIGURE II-2 Mode of Attachment of Glycosyl-Phosphatidylinositol Anchor

cleavage sites for the C-terminal hydrophobic domain of G-PI anchored proteins occur 10-12 residues N-terminal to the C-terminal signal peptide (31). It is believed that the hydrophobic C-terminal signal peptide anchors the protein in the endoplasmic reticulum membrane and that a transpeptidase type of enzyme cleaves the hydrophobic signal peptide and adds the G-PI anchor (28). The G-PI anchor is attached less than 1 minute after the completion of protein synthesis (32).

The rapid addition of the G-PI anchor suggests that it exists in a preassembled form (4, 29). A glycolipid, glycolipid A, has been found in Trypanasoma brucei which has properties consistent with it being an anchor precursor (33, 34). Glycolipid A can be radiolabeled with glucosamine, mannose, ethanolamine, phosphate, and myristate and is susceptible to cleavage by G-PI-specific phospholipases (29). A scheme for the biosynthesis of glycolipid A is shown in Fig. II-3 (adapted) (35). N-acetyl glucosamine is transferred from UDP-N-acetylglucosamine to PI. N-acetylglucosamine PI is then deacylated followed by the addition of mannose residues and incorporation of phosphoethanolamine into the anchor. Fatty acid remodelling occurs to change all the fatty acids to myristic acid and form glycolipid A.

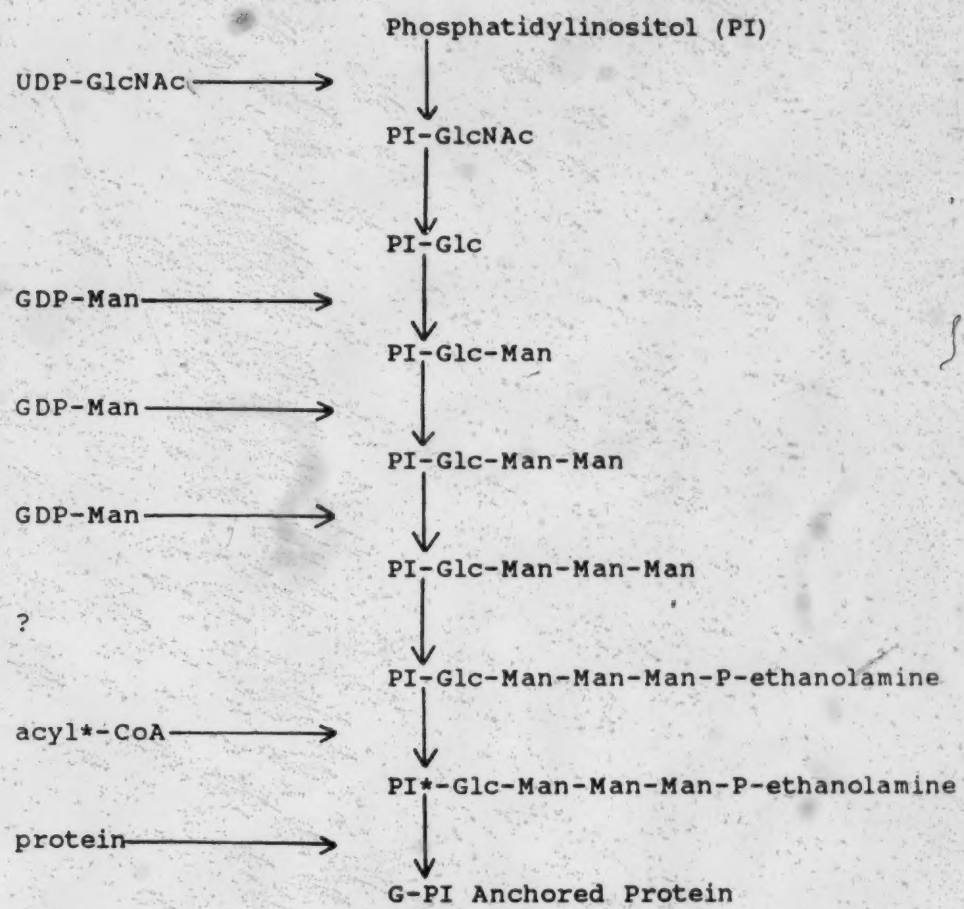

FIGURE II-3 Biosynthesis of the Glycosyl-Phosphatidylinositol Anchor. GlcNac represents N-acetylglucosamine, Man represents mannose, P-ethanolamine represents ethanolamine phosphate

C. FUNCTIONS OF THE GLYCOSYL-PHOSPHATIDYLINOSITOL ANCHOR

The most obvious of these is to anchor proteins in the membrane, but the uniqueness of this anchor has brought about speculation as to other possible functions. The lateral mobility of ALP in plasma membranes of osteoblasts was found to be approximately 10x faster than peptide anchored membrane proteins, and closer to resembling that of membrane lipids (36). The functional significance of this increased mobility is not known.

Another proposed role of the G-PI anchor is based on the discovery of phospholipases with specificity for G-PI anchored proteins. Cleavage by a glycosyl-phosphatidylinositol specific phospholipase (G-PI-PL) would expose the glycan on the soluble protein which may serve as a recognition and binding site for a specific receptor (1). This specific cleavage of G-PI anchored proteins by phospholipases allows trypanosomes to readily shed their coat of VSG in order to avoid the immune system of their host (37). Phospholipase cleavage of G-PI anchored proteins also leads to the production of second messengers such as 1,2-diacylglycerol, phosphatidic acid, and glycosyl-inositol phosphate, which provides the cell with a mechanism for coordinating expression of a cell surface protein with intracellular metabolism (1, 38, 39).

D. PHOSPHOLIPASES-GENERAL

Phospholipases are esterases which can be divided into acyl hydrolases and phosphodiesterases. The acyl hydrolases consist of phospholipases A_1 and A_2 and the phospholipases B or the lysophospholiases, whereas phospholipases C and D are phosphodiesterases. Figure II-4 (adapted) shows the site of cleavage for the different phospholipases (40). Phospholipase A_1 hydrolyzes the SN-1 acyl side chain whereas phospholipase A_2 hydrolyzes the SN-2 acyl side chain. Phospholipases C and D both hydrolyze the SN-3 phosphodiester bond, with diacylglycerol and the phosphorylated polar head group being produced by the C enzyme and phosphatidic acid and the free polar head group being produced by the D enzyme (41 42). Phospholipases exhibit vast differences in substrate specificity, and in molecular and catalytic properties.

Phospholipase A_1 enzymes are widely distributed in nature, being found in prokaryotic and eukaryotic cells, whereas phospholipase A_2 enzymes are predominantly found in bee, scorpion, and snake venoms (40). Phospholipase C enzymes are found in bacteria, yeast, plants, and mammalian tissues, whereas phospholipase D enzymes are found in higher plants, algae, bacteria, and mammalian tissues. Lysophospholipases are widespread in

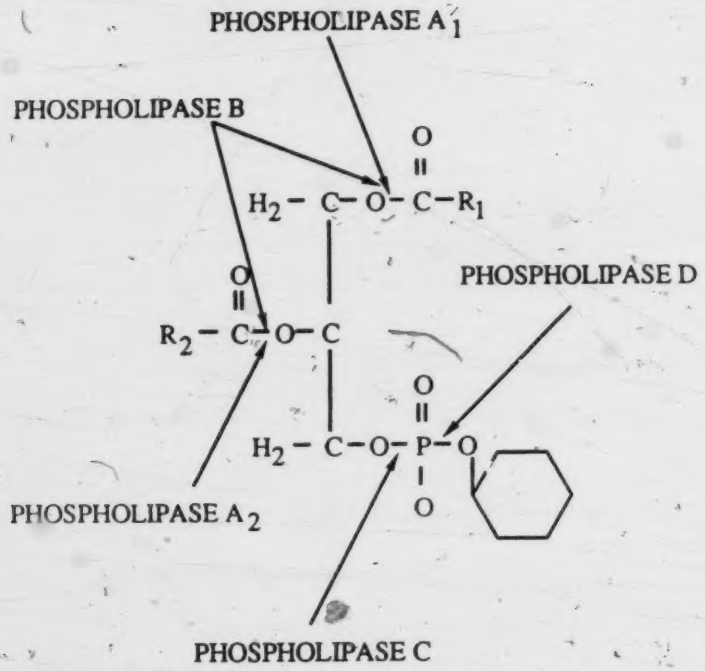

FIGURE II-4 Sites of Cleavage for Phospholipase

nature, being found in prokaryotic and eukaryotic microorgainisms and all other eukaryotic cells.

E. PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C AND D ENZYMES

Bacterial acylhydrolases are generally cell-associated whereas bacterial phosphodiesterases are mainly secreted from the cell in a soluble form, (41) which has greatly facilitated their purification and characterization. Phospholipase C enzymes with specificity for phosphatidylinositol have been purified from Clostridium novyi, Bacillus cereus, Bacillus thurienginsis, and Staphylococcus aureus (43, 44, 45, 46). B. cereus produces three phospholipases with three different phospholipid specificities (47). The phospholipase C that was active against PI was also capable of releasing ALP from bone slices. Streptomyces chromofuscus and Streptomyces hachijoensis are the only two bacteria which contain phospholipase D enzymes with activity towards PI although they show an increased preference for phosphatidylcholine.

Most of the phospholipase C enzymes found in mammalian tissues show specificity for PI or its phosphorylated derivatives (41). These enzymes play an important role in initiating cellular responses through

the PI cycle. It is believed that at least seven PI-PLC isoenzymes exist in mammals, in both soluble and membrane forms, based on M_r and antigenic properties (42, 48).

The emergence of a new group of phospholipases, which are specific for G-PI, has occurred in the last 10 years. Neutral nonionic detergents solubilize a G-PI-PL from rat liver plasma membranes which is capable of releasing diacylglycerol from variant surface glycoprotein (49). G-PI-PLC from rat liver plasma membranes showed no activity towards PI, phosphatidylinositol diphosphate, or phosphatidylcholine and was calcium independent. A similar G-PI-PLC, which has been discovered in the membrane of Trypanosoma brucei is capable of cleaving the anchor of several G-PI anchored proteins suggesting that the specificity is for the G-PI moiety and not for the protein itself (3, 37). Membrane disruption is required to remove VSG from the membrane in trypanosomes, suggesting that the enzyme may be under strict regulation and is only activated during certain stages of the parasites life cycle (50).

F. GLYCOSYL-PHOSPHATIDYLINOSITOL PHOSPHOLIPASES D

An enzyme capable of removing the anchor from ALP in kidney microsomal membranes was discovered during a butanol extraction of this tissue (6). A similar effect

was found in rat liver membranes where acidic pH greatly enhanced this anchor-degrading enzyme (51). Two forms of ALP resulted; one that was able to bind to phospholipid vesicles and one that was not. Increasing the time of the butanol extraction resulted in an increase in the ALP that did not bind to vesicles. EDTA inhibited this anchor removing enzyme, which led to the suggestion that it was the calcium-dependent PI-PLC found in the cytoplasm and that its ability to remove the membrane anchor of ALP was due to cell-lysis and membrane disruption caused by the butanol. Improved techniques for measuring enzymes that remove G-PI anchors from proteins resulted from the use of human placental ALP as a substrate (52). The enzyme removing the anchor of G-PI proteins in numerous tissues was found to be different from the cytoplasmic PI-PLC on the basis of molecular size, sensitivity to heat inactivation, and sensitivity to inhibition by 1,10-phenanthroline (52, 53). The availability of (^{3}H)myristate-labelled VSG as a substrate allowed for the analysis of the cleaved anchor product which was found to be ^{3}H]phosphatidic acid rather than (^{3}H)1,2-diacylglycerol.

An identical or closely related enzyme was also found to be present in plasma or serum of numerous mammalian species (53, 54, 55). This plasma enzyme has a M_r of 110 000 as determined by sodium dodecyl sulfate

polyacrylamide electrophoresis, is susceptible to inhibition by 1,10-phenanthroline and the thiol-blocking agent p-chloromercuriphenylsulfonic acid, (53, 56) and is stimulated by butanol. Plasma G-PI-PLD does not release decay accelerating factor or ALP from intact cells and thus its physiological role is uncertain (56).

It is assumed that the enzyme activity in mammalian tissues comes from the plasma, although there is no evidence on the origin or destination of the plasma enzyme (3). The liver may synthesize and secrete the G-PI-PLD into the plasma for transportation to other tissues, or many tissues may synthesize and secrete the G-PI-PLD and the activity in plasma simply represents enzyme being removed from cells and therefore is not physiologically relevant (3).

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PURPOSE OF STUDY

In the early 1980s, enzyme activities capable of removing the glycosyl-phosphatidylinositol anchor of proteins during a butanol extraction at acidic pH were discovered in mammalian tissues. The same observations were made on the results of butanol extractions on human liver plasma membranes performed in our laboratory. The literature suggested that these enzyme activities were phospholipases with specificity for proteins anchored by glycosyl-phosphatidylinositol. The purpose of this study was to characterize the enzyme activity in human liver plasma membranes that converted hydrophobic alkaline phosphatase to a hydrophilic form, and establish its identity with respect to known phospholipases capable of cleaving glycosyl-phosphatidylinositol anchored proteins. More specifically, it was necessary to solubilize the enzyme from the plasma membranes and develop a reliable assay protocol. With these aims accomplished, a thorough characterization was possible. While this work was in progress a report was published on a glycosyl-phosphatidylinositol phospholipase D from mammalian plasma. There were many similarities with the enzyme in liver plasma membranes and further characterization was undertaken to determine a possible

identity. Subsequently, some aspects of the tissue distribution of the enzyme were determined in an effort to elucidate the site of action and physiological significance of the enzyme.

CHAPTER THREE

PLASMA MEMBRANES FROM HUMAN LIVER CONTAIN AN INTEGRAL MEMBRANE PROTEIN CAPABLE OF CLEAVING THE HYDROPHOBIC ANCHOR OF ALKALINE PHOSPHATASE.

A. INTRODUCTION

Alkaline phosphatase (ALP) is known to be anchored in the membrane by covalent linkage to glycosyl-phosphatidylinositol, a mechanism that renders the enzyme susceptible to release from the membrane by bacterial phosphatidylinositol specific phospholipase C (PI-PLC) enzymes (1). PI-PLC purified from Bacillus cereus not only accomplishes this but also converts a purified hydrophobic form of ALP into a hydrophilic form (2). In this process phosphatidylinositol is hydrolyzed from the C-terminus of ALP releasing diacylglycerol. A glycosyl-phosphatidylinositol-specific phospholipase capable of removing the hydrophobic anchors from the membrane forms of variant surface glycoprotein (mfVSG), decay accelerating factor, ALP, and 5'-nucleotidase has been reported in mammalian plasma (Chapter 2, Section G) (3,4). Evidence suggests that it is a phospholipase D. More recently we have demonstrated the presence of an endogenous enzyme in most human tissues, cells, and

fluids containing ALP, that releases the phosphohydrolase as hydrophilic dimers (5).

Butanol extraction of plasma membranes from human liver at pH 6.0 results in solubilization of hydrophilic ALP, whereas butanol extraction at pH 8.0 results in solubilization of a hydrophobic form (6,7). The hydrophilic ALP produced at pH 6.0 may be due to the action of an endogenous phopholipase, that prefers an acidic pH and is possibly activated by butanol, cleaving the hydrophobic anchor from the enzyme (8). Herein we report on the characterization of such a phospholipase solubilized from the plasma membrane of human liver.

B. MATERIALS AND METHODS

1. Materials

Butanol (1-butanol), 2-butanol, propanol (1-propanol), 2-propanol, ethanol, and sodium chloride were obtained from BDH Inc., Darmstadt, FRG. Octyl-Sepharose CL-4B and phenyl-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), p-nitrophenyl phosphate, boric acid, naphthol AS-MX phosphate, Triton X-100, Triton X-114, Nonidet P-40 (NP-40), deoxycholate, cholate, β-D-octyl glucoside,

2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), Tris, 1,10-phenanthroline, and p-nitrophenylphosphorylcholine were purchased from Sigma Chemical Company, St. Louis, MO, USA and L-3-phosphatidyl(2-^{3}H)inositol from Amersham Canada Limited, Oakville, ON, Canada. The buffer 2-(ethylamino)ethanol (gold label) was purchased from the Aldrich Chemical Company, Inc., Milwaukee, WI, USA, acrylamide, bis-acrylamide, sucrose, TEMED, ammonium persulfate, molecular weight standards, and a Bio-Sil 4000SW HPLC column from Bio-Rad Laboratories, Richmond, CA, USA, and magnesium chloride, calcium chloride, manganese chloride, zinc chloride, and pentanol (1-pentanol) from Fisher Scientific Company, Fair Lawn, NJ, USA. Mouse anti-human alkaline phosphatase monoclonal antibody, OST 6, produced as described previously (9,10) was immobilized onto CNBr activated Sepharose 4B (11).

2. Alkaline Phosphatase Assay

Alkaline phosphatase activity was assayed at 30°C in 1 ml of medium containing 10 mmol/l p-nitrophenylphosphate, 1.5 mmol/l $MgCl_2$ and 1.0 mol/l 2-(ethylamino)ethanol, pH 10.3. The increase in absorbance at 404 nm was monitored with a spectrophotometer (Varian, model 2200 or Perkin-Elmer,

model Lambda 3A), and enzyme activity was expressed as p-nitrophenol released (μmol/min/l) (12).

3. Purification of Hydrophobic Liver and Placental Alkaline Phosphatases

Hydrophobic liver ALP was purified by modification of a previously published protocol (7). The aqueous layer from a butanol extraction was recovered, centrifuged at 140,000 x g for 45 min at 4°C, and applied to a phenyl-Sepharose CL-4B column (1.6 cm x 20 cm) equilibrated with 50 mmol/l Tris, pH 8.5. Subsequent to washing, the ALP was eluted with 50 mmol/l Tris, pH 8.5, 5% (v/v) Triton X-100, and then applied to a column of immobilized monoclonal antibody to ALP. The ALP was eluted with 0.1 mol/l 2-amino-2-methyl-1-propanol (AMP), pH 10.3, plus 0.1% (v/v) Triton X-100 and then applied to 1.0 ml of packed phenyl-Sepharose. The phenyl-Sepharose was washed with 50 mmol/l Tris, pH 7.5 and the ALP was eluted with 50 mmol/l Tris, pH 7.5, 5% (v/v) Triton X-100. This second treatment with phenyl-Sepharose allowed removal of the AMP, lowering of the pH, and concentration of the enzyme. Hydrophobic placental ALP was purified as described previously (2).

4. Detergent Solubilization of an Activity in Liver Plasma Membranes that Converts Hydrophobic ALP to Hydrophilic ALP

Plasma membranes were prepared from healthy human liver and stored at -20°C (13). Routinely, membranes were thawed and resuspended in 50 mmol/l MES, pH 6.0, 1% (v/v) Triton X-100 and incubated on ice for 1 h, with gentle vortexing every 15 min. The mixture was centrifuged at 14 000 x g for 5 min at room temperature and the supernatant applied to a column of immobilized monoclonal antibody to liver ALP. This was necessary to remove endogenous phosphatase activity. The void containing the alkaline phosphatase converting activity (ALPCA) was recovered. ALPCA refers to the enzyme activity, found in liver plasma membranes or solubilized from these membranes, that converts hydrophobic ALP to a hydrophilic form.

The ability of several detergents to solubilize the ALPCA was tested by resuspending plasma membranes in 50 mmol/l MES, pH 6.0 in the presence of 1% Triton X-100, 1% Triton X-114, 1% NP-40, 48 mmol/l cholate, 10 mmol/l CHAPS, 7 mmol/l deoxycholate, or 60 mmol/l β-D-octylglucoside in the same manner as described above for Triton X-100, and then performing the ALPCA assay. These detergent concentrations were selected so as to be above their respective critical micelle

concentration. Alkaline phosphatase endogenous to the plasma membranes was solubilized by these detergents but was not removed prior to assay of ALPCA.

The ability of detergents to activate ALPCA was tested by adding detergents, at the concentrations listed above, to 63 µl of ALPCA that had been solubilized from the plasma membranes with 1% (v/v) Triton X-100. Hydrophobic placental ALP (112 µl of 3000 U/l) was then added and the mixture was incubated at 30°C for 2 h. The percent conversion to hydrophilic ALP was measured by adsorption to phenyl-Sepharose CL-4B. Detergents were also tested for there ability to activate ALPCA in serum by adding detergents at the concentrations listed above to 63 µl of serum, diluted 1:250 in 50 mmol/l MES, pH 6.0, and then adding 112 µl of hydrophobic placental ALP and incubating at 30°C for 15 min.

5. Assay of Alkaline Phosphatase Converting Activity (ALPCA)

This was carried out by determining the rate of conversion of hydrophobic ALP to a hydrophilic form; the former adsorbs to octyl-Sepharose CL-4B whereas the latter does not.

A mixture of 63 µl of Triton-solubilized ALPCA and 112 µl of butanol extracted (to remove Triton)

hydrophobic liver ALP (3000 U/l) in 50 mmol/l MES, pH 6.0 were incubated at 30°C. At various times, 150 µl of the above mixture was removed and the reaction stopped by addition of 100 µl of 250 mmol/l Tris, pH 9.0. To permit adsorption of the remaining hydrophobic ALP to octyl-Sepharose the Triton X-100 was removed by butanol; 250 µl cold butanol was added to the above mixture. After 10 min with vortexing every 2 min, this was centrifuged at 14000 x g for 5 min. The aqueous layer was removed and the percent conversion to hydrophilic ALP was determined by adsorption of hydrophobic ALP to octyl-Sepharose CL-4B (5). The amount of hydrophobic ALP converted to a hydrophilic form was expressed as a percent of the total ALP activity. For time course experiments ALPCA was assayed every 2 h over 8 h using either hydrophobic liver or placental ALP as substrate.

For slab gradient gel (2.5-27%) electrophoresis in polyacrylamide, the ALPCA assay was stopped by the addition of 250 mmol/l Tris, pH 9.0 and 100 µl applied to the gel (14). The ALP activity was located by staining with naphthol AS-MX phosphate and photographed under ultraviolet light (15).

6. Determination of the Bond Specificity of ALPCA

The bond specificity of ALPCA was determined by examination of the protein hydrolysis products of

placental ALP electrophoretically. ALPCA (40 µl) was incubated with 70 µl of pure placental alkaline phosphatase (1500 U/l) in the presence of 1 mmol/l levamisole for 4 h at 30°C. Serum and PI-PLC from B. cereus were also incubated with placental ALP as phospholipase D and C controls, respectively. The reaction was stopped with 125 µl of 250 mmol/l Tris, pH 9.0 and 40 µl was applied to a 7% (w/v) polyacrylamide electrophoresis gel with a 4% (w/v) polyacrylamide stacking gel and electrophoresed for 4 h at 30 mA. The electrophoresis buffer consisted of 90 mmol/l Tris and 90 mmol/l boric acid, pH 8.4. The gel buffer was 1.5 mol/l Tris, pH 8.8. Triton X-100 (0.1% v/v) was present in the gel and buffers. The ALP activity was located by staining with naphthol AS-MX phosphate and photographed under ultraviolet light (15)

7. The Effect of Alcohols on the ALPCA

The effects of ethanol, propanol, 2-propanol, butanol, 2-butanol, and pentanol on Triton-solubilized ALPCA activity were examined by performing the 2 h assay in the presence of each alcohol at a concentration of 1 mol/l, except for pentanol whose solubility limited its concentration to 375 mmol/l.

Time courses with Triton-solubilized ALPCA and hydrophobic liver ALP in the presence of saturating

amounts of butanol (1 mol/l) or pentanol (375 mmol/l) were for 2 h with assays every 30 min. Controls contained no ALPCA. Time courses were also carried out for ALPCA in intact liver plasma membranes in the presence of saturating amounts of butanol and pentanol. Liver plasma membranes were resuspended in 50 mmol/l MES, pH 6.0. Butanol at 1 mol/l or 375 mmol/l pentanol were then added to the membranes and the mixture was incubated at 30°C. At selected time periods, 250 µl of solution were removed and centrifuged for 5 min at 14 000 x g. The hydrophobic ALP in the aqueous layer was adsorbed to octyl-Sepharose and the percent conversion to hydrophilic ALP was determined.

8. Heat Stability and Effect of Inhibitors on ALPCA

Triton-solubilized ALPCA was treated at 70°C for various time periods and assayed as above. Controls were kept on ice or boiled for 5 min.

The ALPCA assay was performed in the presence of 1 mmol/l leupeptin, or pepstatin A, or phenylmethylsulfonyl fluoride (PMSF), or a mixture of the three protease inhibitors. ALPCA and the inhibitors were incubated for 5 min followed by a 4 h incubation with hydrophobic liver ALP (3000 U/l). Controls did not contain protease inhibitors.

To assess the effect of 1,10-phenanthroline, this

zinc chelator at 200 μmol/l was incubated with ALPCA for 3 h on ice; for some samples this was followed by a 5 min incubation on ice with 205 μmol/l $ZnCl_2$. All samples were then incubated with hydrophobic ALP at 30°C for 4 h. The effect of 1,10-phenanthroline on ALPCA in intact membranes was measured by incubating plasma membranes with 300 and 500 μmol/l of the chelator for 10 min and then adding an equal volume of butanol to the plasma membranes. This mixture was incubated at 30°C for 1 h and the percent conversion to hydrophilic ALP was measured by octyl-Sepharose adsorption. Controls contained no 1,10-phenanthroline.

9. pH Profiles

Plasma membranes in 10 mmol/l MES, pH 6.0 were solubilized with 1% (v/v) Triton X-100 for 1 h on ice. The supernatants recovered from centrifugation at 14 000 x g for 5 min were adjusted to various pH values by adding 100 mmol/l MES (pH 5.0-6.5) or 100 mmol/l MOPS (pH 7.0 and 8.0). These supernatants were then mixed with hydrophobic liver ALP at the same pH values.

Alternatively, butanol extractions of plasma membranes were performed at various pH values by resuspending the liver plasma membranes in 50 mmol/l MES (pH 5.25-6.5) and 50 mmol/l MOPS (pH 7.0 and 7.5). Equal volumes of butanol and membranes were mixed and incubated

at 30°C for 30 min. The mixtures were centrifuged at 14 000 x g for 5 min and the percent conversion to hydrophilic ALP was measured in the aqueous layer by adsorption to octyl-Sepharose.

10. Substrate Specificity and Molecular Weight of ALPCA

Hydrophobic placental ALP was evaluated as a substrate for ALPCA. The same assay as described above for liver ALP (Section B-4) was used except that phenyl-Sepharose CL-4B was used to adsorb the hydrophobic placental enzyme. The ability of ALPCA to remove the hydrophobic portion of phosphatidylinositol was assessed by mixing 63 µl of ALPCA with 112 µl of L-3-phosphatidyl(2-^{3}H)inositol (4000 cpm) for 4 h at 30°C. The potential products, inositol phosphate or inositol, were extracted into the aqueous layer following treatment with 250 µl of chloroform/methanol/hydrochloric acid (100/100/0.6) and counted by liquid scintillation. p-Nitrophenylphosphorylcholine was tested as a substrate by incubating 100 µl of ALPCA with 1 ml of 5 mmol/l of the phospholipid derivative in 50 mmol/l MES, pH 6.0 for 4 h at 30°C. The reaction was stopped by the addition of 200 µl of 1.0 mol/l sodium hydroxide and the absorbance read at 404 nm.

ALPCA was tested for its ability to release ALP from

intact liver plasma membranes by incubating 63 µl of ALPCA with 112 µl of liver plasma membranes resuspended in 50 mmol/l MES, pH 6.0 for 4 h at 30°C. The mixture was centrifuged for 5 min at 14 000 x g and the supernatant analyzed for hydrophilic ALP by adsorption to octyl-Sepharose.

The native M_r of the protein with ALPCA was determined as follows. Liver plasma membranes were solubilized for 1 h in 50 mmol/l MES, pH 6.0, 0.25% Triton X-100. The supernatant was concentrated appproximately four times in an Amicon concentration cell using a PM-10 membrane. The concentrated ALPCA and molecular weight standards were then applied as a mixture to a Bio-Sil Sec-400 HPLC column (600 X 7.5 mm). Fractions were analyzed for ALPCA by mixing 63 µl of eluate with 112 µl of hydrophobic liver ALP and 18 µl of butanol. This mixture was incubated for 2 h at 30°C and the percent conversion to hydrophilic ALP was determined by octyl-Sepharose adsorption. To determine a M_r, the elution volume of the ALPCA was compared to the elution volume of the molecular weight standards thyroglobulin (670 000), immunoglobulin G (158 000), ovalbumin (44 000), myoglobin (17 000), and vitamin B_{12} (1350) (16).

11. Distribution of ALPCA

The amount of ALPCA present in whole liver, liver plasma membranes, and serum was expressed as the amount of ALP converted to hydrophilic dimers as mUnits of ALP converted/mg protein/min. ALPCA from plasma membranes was obtained as described above (Section B-4) and serum was used without dilution. ALPCA from whole liver was obtained by homogenizing 2 g of normal human liver tissue (obtained at autopsy and stored at -70°C) in 10 ml of 50 mmol/l MES, pH 6.0. This was followed by solubilization with 1% (v/v) Triton X-100 in the same manner as for the liver plasma membranes.

C. RESULTS

1. Detergent Solubilization of ALPCA

The non-ionic detergents Triton X-100, NP-40, and Triton X-114, all at 1% (v/v), solubilized the ALPCA, with the last two being more efficient (Fig. III-1). The ionic detergents, cholate and deoxycholate, were much less effective solubilization agents, and β-D-octylglucoside and the zwitterionic detergent, CHAPS, were ineffective. Triton X-100 was used for routine solubilization to maintain consistency with related research. Treatment of the liver plasma membranes with 250 mmol/l sodium chloride did not result in the solubilization of any ALPCA.

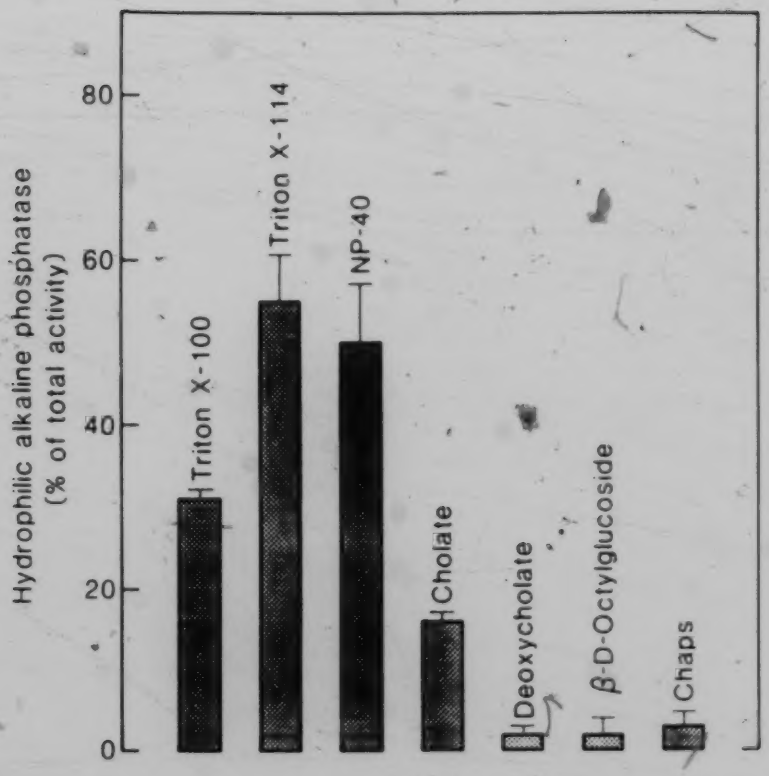

FIGURE III-1 Solubilization of ALPCA by Detergents. This was measured by the ability of the supernatant, following detergent solubilization of plasma membranes, to convert hydrophobic liver ALP to a hydrophilic form. The bars show 1 SD. Detergent concentrations are given in Section B-4.

ALPCA solublized with 1% (v/v) Triton X-100 showed only a small activation by the two detergents that were best at apparent solubilization, Triton X-114 and NP-40 (Table III-1). Deoxycholate and β-D-octylglucoside, which solubilized ALPCA poorly, did not inhibit the Triton-solubilized enzyme whereas cholate, which appeared to solubilize reasonably well, profoumdly inhibited Triton-solublized ALPCA. The non-ionic detergents, Triton, NP-40 and β-D-octylglucoside have either a small inhibitory or no effect on ALPCA in serum. The detergents cholate and CHAPS on the other hand profoundly inhibited the conversion.

2. Time Courses

The time courses for the conversion of both hydrophobic liver and placental ALPs to their respective hydrophilic forms were similar, but the ALPCA had a slight preference for the liver isoenzyme (Fig. III-2). Electrophoresis in a polyacrylamide gradient gel showed increasing amounts of hydrophilic liver ALP and decreasing amounts of the hydrophobic form with incubation time (Plate III-1).

3. Bond Specificity of ALPCA

Electrophoresis of ALPCA treated placental ALP showed a protein species that migrated slower than that

TABLE III-1 THE EFFECT OF DETERGENTS ON TRITON-SOLUBILIZED ALPCA OF PLASMA MEMBRANES AND ALPCA OF SERUM

	Percent Conversion of Hydrophobic Alkaline Phosphatase to a Hydrophilic Form (1 SD)	
Detergent*	Triton-solubilized ALPCA	Serum ALPCA
No Addition	25 (2.8)	62 (5.1)
Triton X-100	25	33 (10.3)
Triton X-113	36 (0.0)	71 (12.5)
NP-40	36 (2.1)	62 (17.1)
Cholate	0 (0.7)	5 (2.1)
Deoxycholate	24 (2.8)	31 (19.0)
β-D-Octylglucoside	22 (4.9)	47 (11.3)
CHAPS	8	18

* Concentrations as for Figure III-1.

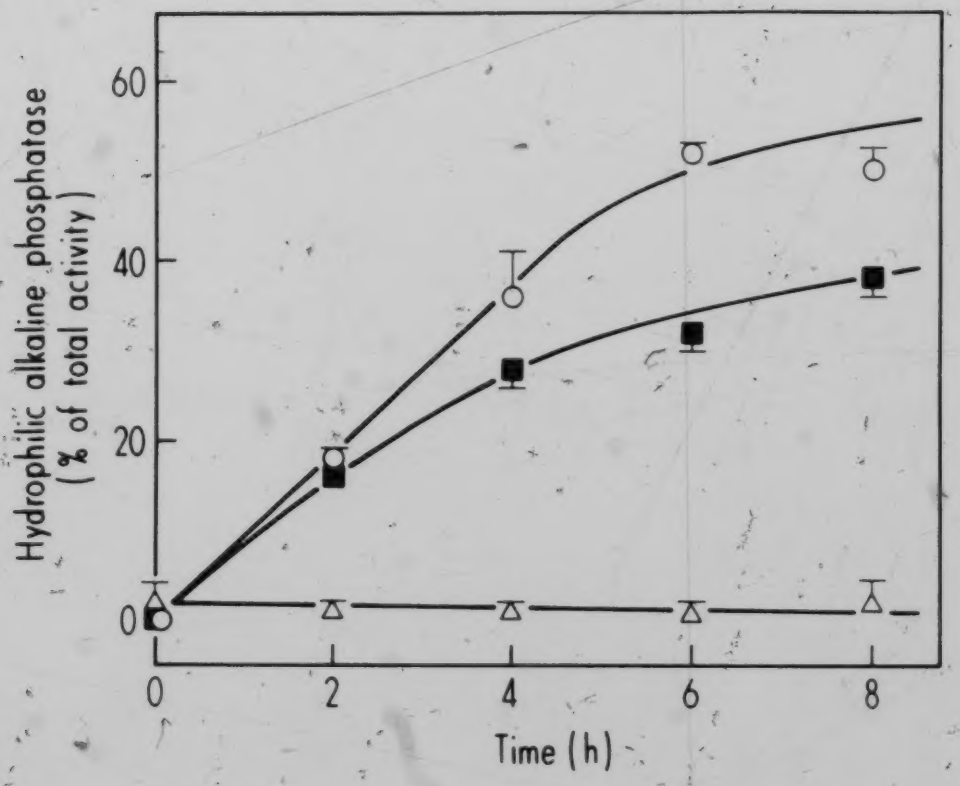

FIGURE III-2 Time Course for ALPCA Using Liver and Placental ALPs as Substrates. Hydrophobic liver (○) and placental (■) forms were converted in 50 mmol/l MES, pH 6.0. Controls (△) did not contain converting activity. The bars show 1 SD.

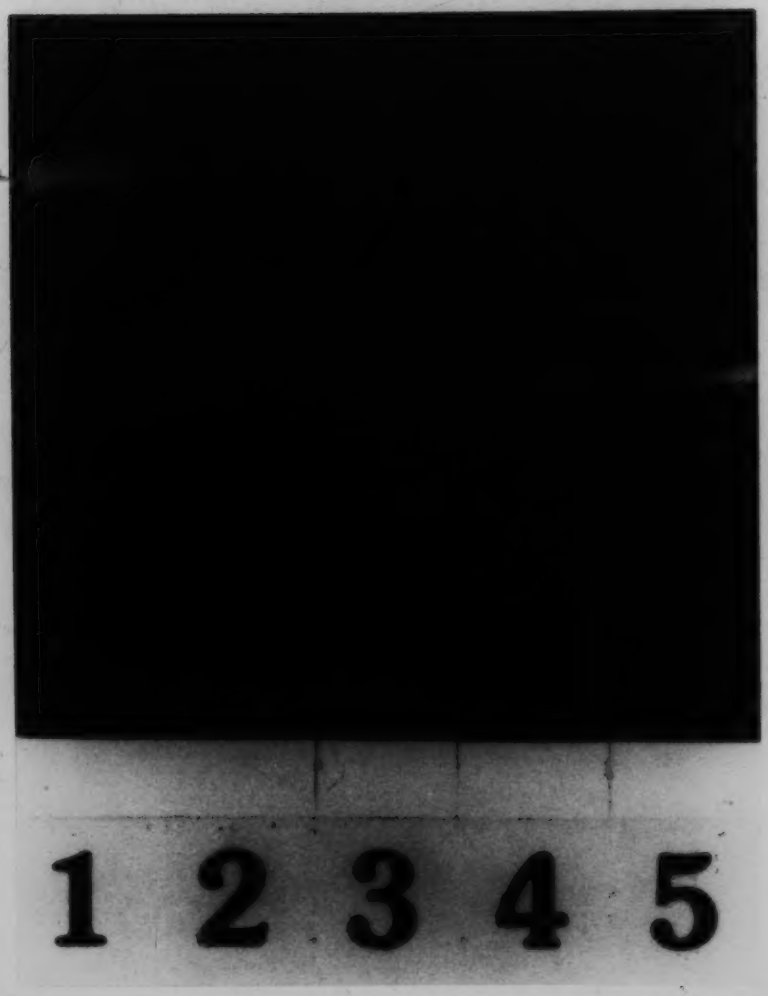

PLATE III-1 Gradient Gel Electropherogram of Products of ALPCA Acting on Hydrophobic ALP. Conversion was at pH 6.0: lane 1, purified hydrophobic liver ALP; lane 2, purified hydrophilic ALP; lane 3, 2 h incubation of ALPCA and hydrophobic liver ALP; lane 4, 4 h incubation of ALPCA and hydrophobic liver ALP; lane 5, 8 h incubation of ALPCA and hydrophobic liver ALP.

obtained by cleavage with PI-PLC but identical with that obtained by treatment with the phospholipase D from human serum (Plate III-2). These mobility differences can be explained by the presence of a phosphate group on the C-terminal end of ALP following cleavage of the anchor with PI-PLC. The absence of this phosphate if treatment is with the phospholipase D of plasma would cause the protein to have a slower migration. The addition of the ALP inhibitor, levamisole, to these incubations was to insure that following cleavage of the glycosyl-phosphatidylinositol, the ALP did not dephosphorylate itself, although this is unlikely at the incubation pH of 6.0. These results suggest that ALPCA has the bond specificity of a phospholipase D.

4. <u>The Effect of Alcohol on ALPCA in Plasma Membranes and in a Triton X-100 Solubilized Form</u>

Butanol and pentanol were the only two alcohols to significantly affect Triton-solubilized ALPCA, although 2-butanol inhibited slightly (Fig. III-3). Treatment of liver plasma membranes with the organic solvents, butanol and pentanol, resulted in disruption of the membrane structure so as to allow endogenous ALPCA to convert hydrophobic ALP to a hydrophilic form. A time course for this activity is Figure III-4. Triton X-100 (1% v/v) treatment of liver plasma membranes was much less

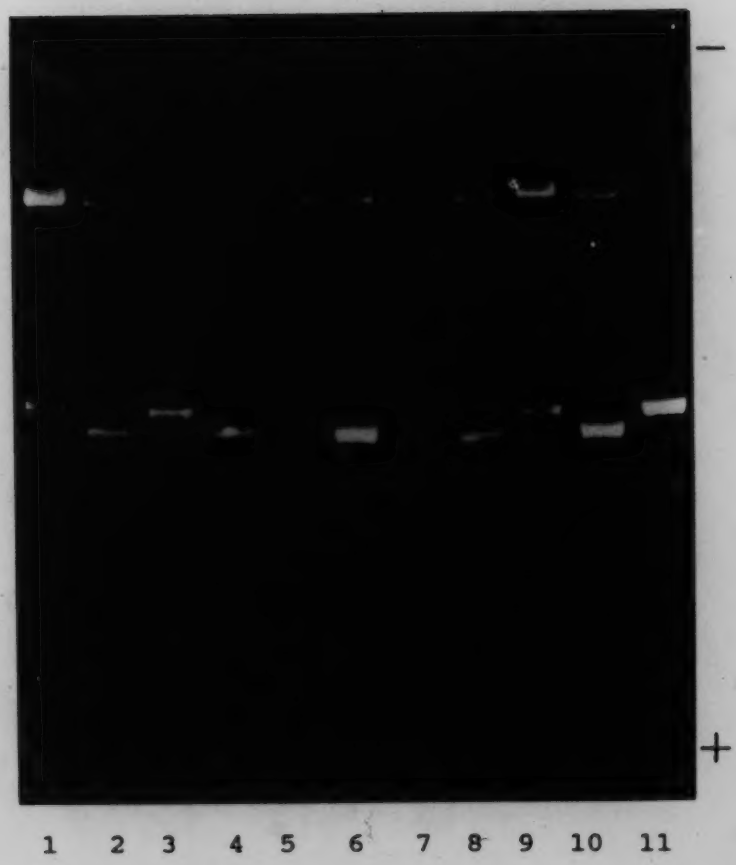

PLATE III-2 Polyacrylamide Electropherogram of Products of Phospholipase Hydrolysis. Lanes 1, 5, 9 - hydrophobic placental ALP treated with ALPCA; Lanes 2, 4, 6, 8, 10 - hydrophobic placental ALP treated with Bacillus cereus PI-PLC; Lanes 3, 7, 11 - hydrophobic placental ALP treated with serum.

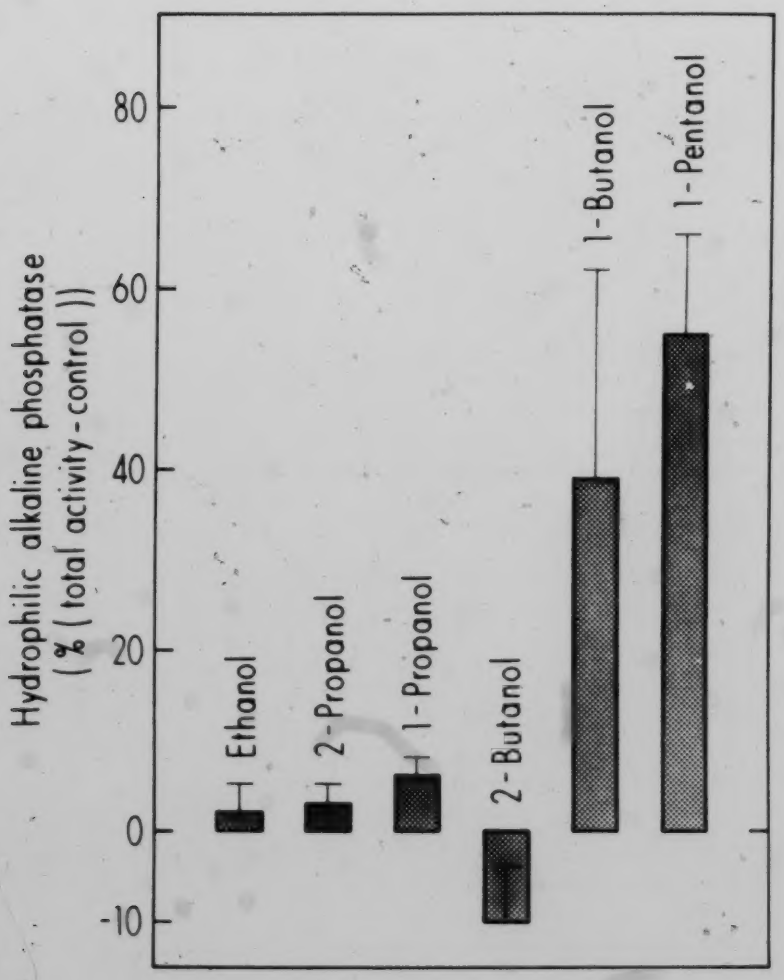

FIGURE III-3 The Effect of Alcohols on the Actiyation of Solubilized ALPCA. The conversion of purified hydrophobic liver ALP to a hydrophilic form by Triton-solubilized ALPCA was measured at pH 6.0 for 2 h. The bars show 1 SD.

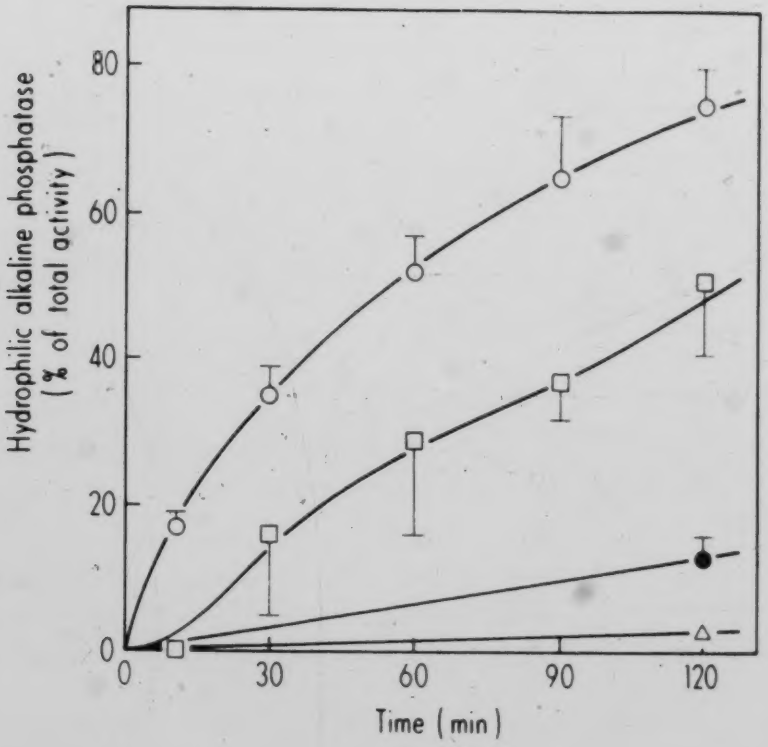

FIGURE III-4 Time Course for the Effect of Butanol and Pentanol on Endogenous ALPCA. Conversion of ALP from liver plasma membranes to a hydrophilic form during treatment with 1 mol/l butanol (○), 375 mmol/l pentanol (□), or 1% (v/v) Triton X-100 (●) of liver plasma membranes at pH 6.0. Controls (△) contained no additions. The bars show 1 SD.

effective at promoting conversion but could be stimulated by alcohols. A time course showing the effect of butanol and pentanol on the ability of Triton X-100-solubilized ALPCA to convert purified hydrophobic ALP to a hydrophilic form is Figure III-5. The activation of Triton-solubilized ALPCA by butanol and pentanol was variable, from 4-10 fold and may account for the increased activation of pentanol over butanol in Figure III-3 and the reverse in Figure III-5.

5. Heat Stability and Inhibitors

ALPCA was relatively heat stable, requiring 60 min to inactivate the conversion by 50% at 70°C (Fig. III-6). Boiling for 5 min completely inactivated the ALPCA (Table III-2).

The protease inhibitors, leupeptin, pepstatin A, and PMSF showed no inhibition of the ALPCA and the latter showed some activation (Table III-2). 1,10-Phenanthroline at 200 μmol/l significantly inhibited the Triton-solubilized ALPCA conversion of hydrophobic liver ALP to a hydrophilic form (Table III-2), but was reversed upon addition of 205 μmol/l $ZnCl_2$. 1,10-Phenanthroline at 500 μmol/l was required to completely inhibit ALPCA in intact plasma membranes during butanol treatment at pH 6.0.

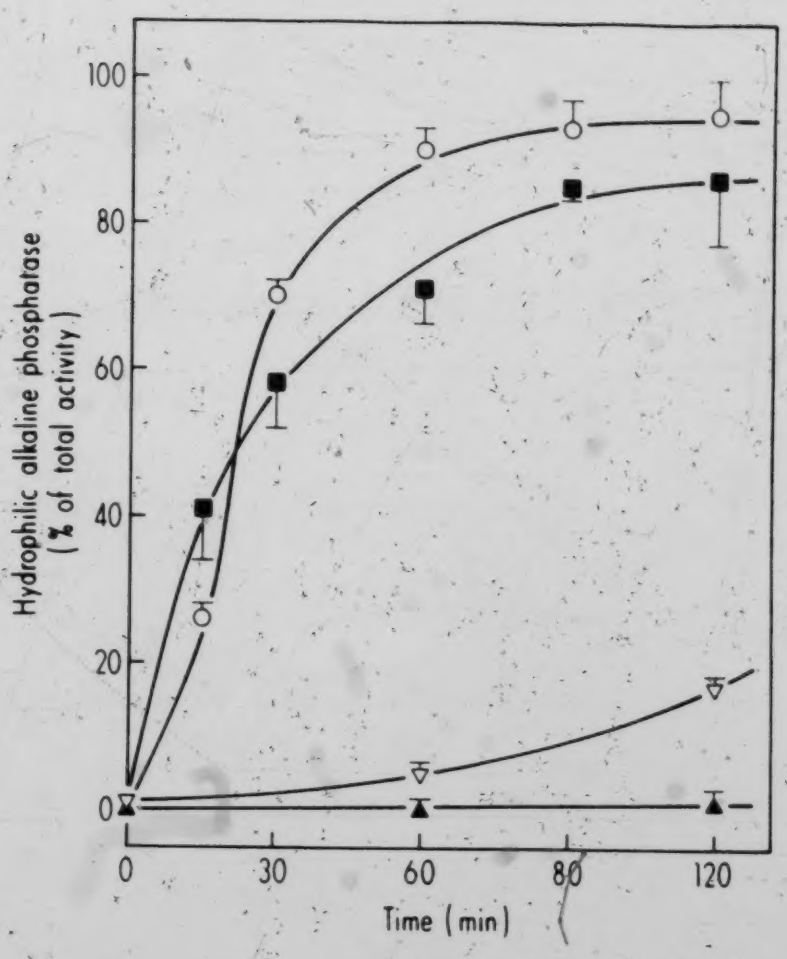

FIGURE III-5 Time Course for the Effect of Butanol and Pentanol on Triton-Solubilized ALPCA. Conversion of purified hydrophobic liver ALP to a hydrophilic form by Triton-solubilized ALPCA in the presence of 375 mmol/l pentanol (■), or 1 mol/l butanol (○), or 1% (v/v) Triton X-100 (▽) at pH 6.0. Conversion in the absence of ALPCA is shown (▲). The bars show 1 SD.

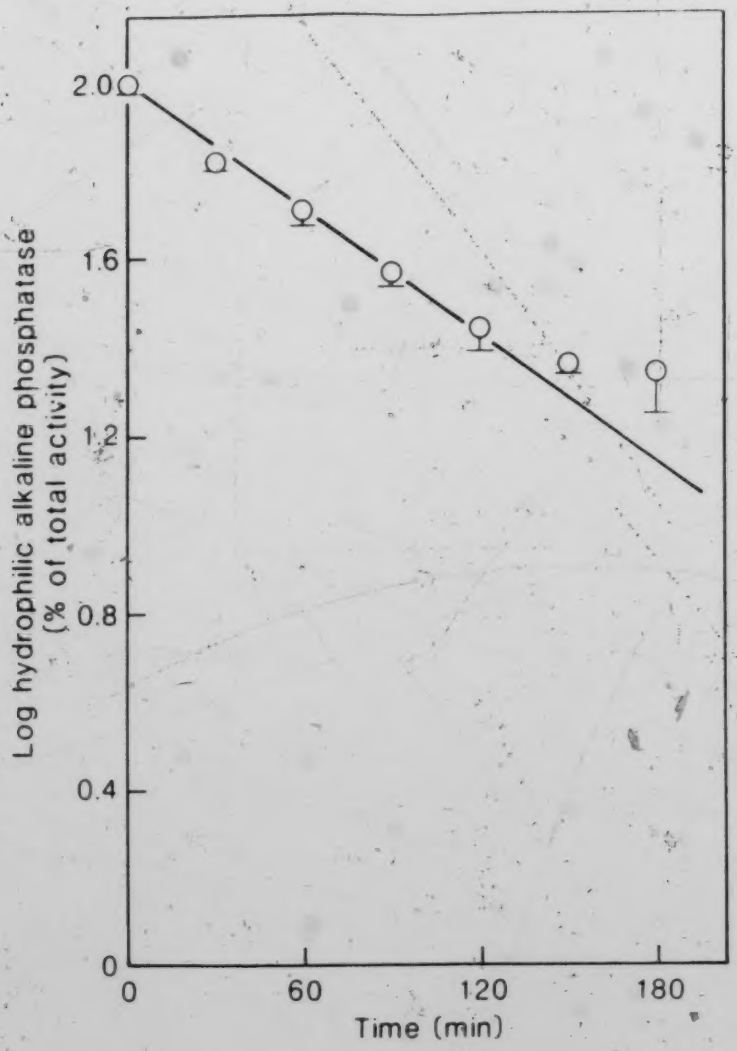

FIGURE II-6 Denaturation of ALPCA at 70°C. The bars show 1 SD.

TABLE III-2 THE EFFECT OF ADDITIONS ON ALPCA*

Condition	Percent Conversion to Hydrophilic Alkaline Phosphatase (1 SD)
Control	100(4.5)
Leupeptin (1 mmol/l)	108(5.2)
Pepatatin A (1 mmol/l)	94(2.9)
PMSF (1 mmol/l)	136(2.8)
56°C (2 h)	117(14.3)
Boiling (5 min)	4(1.5)
1,10-phenanthroline (200 μmol/l)	58(2.0)
1,10-phenanthroline (200 μmol/l) + $ZnCl_2$ (205 μmol/l)	90(2.0)
Butanol extraction of plasma membranes + 300 μmol/l 1,10-phenanthroline	64(5.1)
Butanol extraction of plasma membranes + 500 μmol/l 1,10-phenanthroline	2(2.1)

*The substrate was purified hydrophobic liver ALP in 50 mmol/l MES, pH 6.0.

6. pH Profiles

The optimum pH for ALPCA in a butanol extraction of liver plasma membranes was 5.5 (Fig. III-7). The pH profile for the Triton-solubilized ALPCA conversion of hydrophobic liver ALP to a hydrophilic form exhibited an optimum at approximately pH 6.0 (Fig. III-7). The pH profile for Triton-solubilized ALPCA indicates that the ALPCA may be active below pH 5.0 but this could not be studied due to the instability of ALP at lower pH values. Both pH profiles show the ALPCA has low activity at pH values greater than 8.0.

7. Substrate Specificity and Molecular Weight of ALPCA

Triton-solubilized ALPCA was able to convert both hydrophobic liver and placental ALPs to hydrophilic forms. It acted on membrane-bound ALP only when the membranes were first disrupted with either detergent or with an organic solvent. No hydrolysis of p-nitrophenylphosphorycholine, or L-3-phosphatidyl(2-^{3}H)inositol) was detected.

The ALPCA was found to have a molecular weight of 120 000 ± 14 000 when chromatographed on a Bio-Sil SEC-400 HPLC column in the presence of 1% (v/v) Triton X-100 (Fig. III-8).

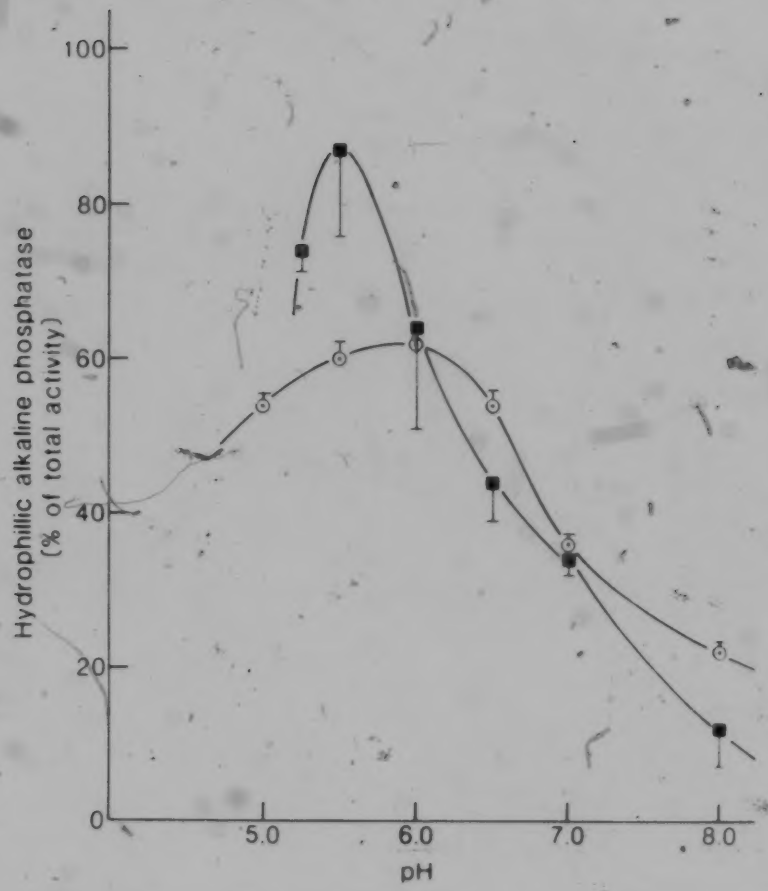

FIGURE III-7 The Effect of pH on ALPCA in Butanol-Extracted Plasma Membranes or on Triton X-100 Solubilized ALPCA. pH Profiles were determined for the activation of ALPCA in plasma membranes by butanol (1 mol/l) acting on endogenous ALP (■), and for Triton-solubilized ALPCA acting on purified liver ALP (⊙). The bars show 1 SD.

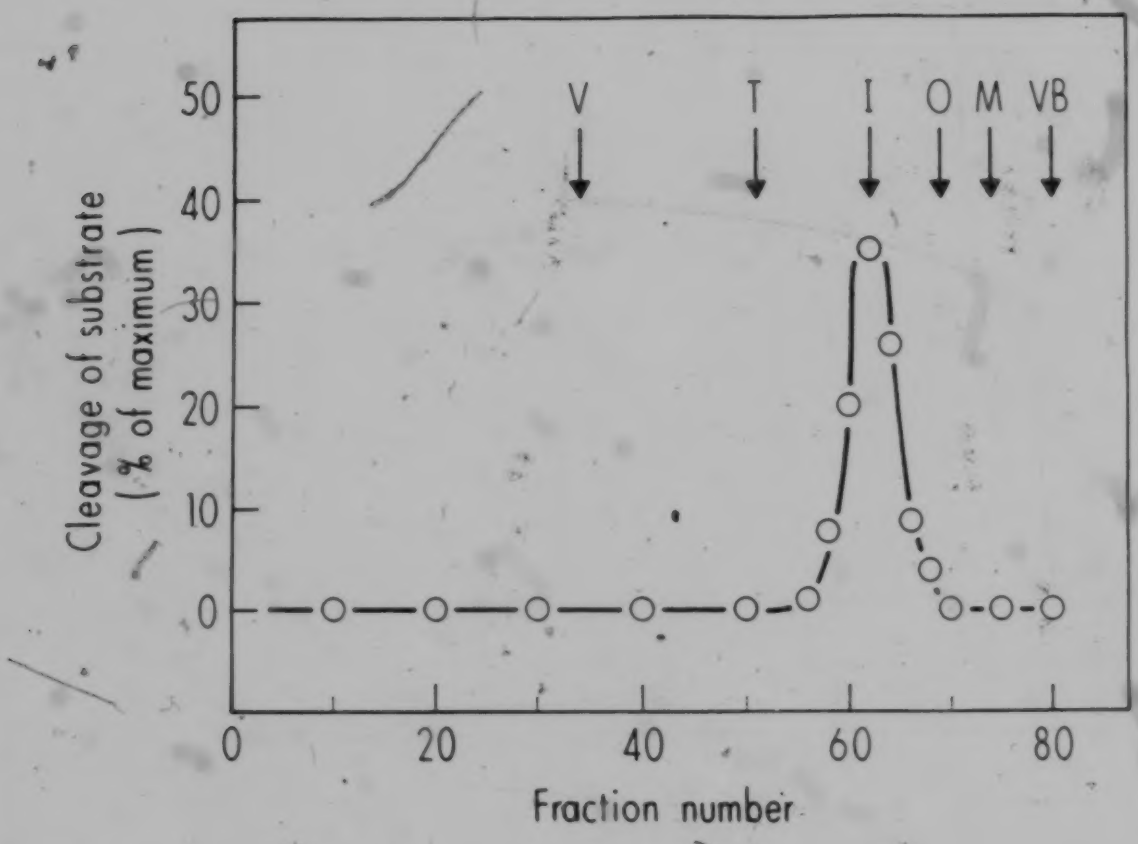

FIGURE III-8 HPLC Gel Filtration of ALPCA. The arrows indicate the elution points of the molecular weight standards; V-void; T-thyroglobulin (670 000); I-immunoglobulin G (158 000); O-ovalbumin (44 000); M-myoglobin (17 000); VB-vitamin B_{12} (1350).

8. Distribution of ALPCA

The amount of ALPCA as a percent of total protein in liver plasma membranes was approximately the same as that in whole liver (Table III-3), whereas serum had approximately 10x more ALPCA.

D. DISCUSSION

Non-ionic detergents with low critical micelle concentrations were the most effective at solubilizing the ALPCA in an active form (Fig. III-1). The three non-ionic detergents, Triton X-100, Triton X-114, and NP-40 have critical micelle concentrations that are at least 10 times lower than the other detergents used (17). Non-ionic detergents, such as Triton X-100, are considered to be the most effective agents for solubilizing integral membrane proteins in their native conformation and in general cause less inactivation than other detergents such as the bile salts (18, 19). This may explain why only the non-ionic detergents with low critical micelle concentrations were effective in solubilizing the ALPCA in an active form. It is difficult to absolutely rule out the possibility that ALPCA could have adsorbed to the plasma membrane of the intact liver from circulating plasma, where high concentrations have been shown to exist. However,

TABLE III-3 DISTRIBUTION OF ALKALINE PHOSPHATASE CONVERTING ACTIVITY

Tissue or fluid	mUnits of ALP converted/ mg protein/min (1 SD)
Whole Liver	20 (6.3)
Liver Plasma Membranes	21 (8.6)
Serum	190 (51)

preparation of the plasma membranes does involve sonication and density gradient centrifugation and thus any loosely bound protein would likely be stripped off. Further, treatment of the isolated plasma membranes with 250 mmol/l sodium chloride failed to release any ALPCA. Also, plasma membranes exhibit ALPCA activity only when disrupted, and thus the activity is unlikely to be a result of nonspecific adsorption to the exterior of the cell. This evidence strongly supports integral plasma membrane location for ALPCA.

Based on the effects of detergents on ALPCA from Triton-solubilized plasma membranes and in serum (Table III-1), it is likely that the data of Figure III-1 represent a combination of the differential solubilization and activation/inhibition properties of the detergents. In particular, β-D-octylglucoside, the poorest detergent at solubilization at apparent solubilization, had little effect on ALPCA in serum of in Triton-solubilized plasma membranes, and cholate whose apparent solubilization was good, profoundly inhibited both sources of the enzyme.

The ability of alcohols to activate a phosphoinositidase in turkey erythrocyte membranes has been shown to be related to their hydrophobicity (20). The more hydrophobic the alcohol the greater the ability to partition into the membrane or promote a specific

hydrophobic domain. The same phenomenom may be occurring in our system. Butanol and pentanol were the only alcohols to activate the ALPCA significantly and were the only alcohols employed with partition coefficients greater than one (21).

The effect of butanol and pentanol on the ALPCA during extractions of liver plasma membranes may be two-fold (Fig. III-4); the alcohols may be both solubilizing and activating endogenous ALPCA. The fact that these two alcohols enhance Triton-solubilized ALPCA suggests that they are also involved by an unknown mechanism in direct activation of ALPCA (Fig. III-5). Butanol has a larger effect than did pentanol on plasma membrane ALPCA, whereas there was little difference between the two alcohols when Triton-solubilized ALPCA was used. Butanol may be a better solubilizing agent for ALPCA but both alcohols activate the enzyme to a similar degree. Triton X-100 treatment of liver plasma membranes had little effect on allowing endogenous ALPCA to convert hydrophobic ALP to a hydrophilic form. It may be possible that the anchor on ALP endogenous to plasma membranes is not accessible to the ALPCA due to bound lipids or proteins and that it only becomes accessible following an alcohol extraction. The small difference in pH optimum for ALPCA between a butanol treatment of plasma membranes and Triton-solubilized ALPCA may result

from the different methods of solubilization and/or a pH dependence of the activation by butanol.

The substrate specificity of ALPCA suggests that it may be glycosyl-phosphatidylinositol specific. ALPCA shows no activity towards phosphatidylinositol alone but converts both hydrophobic liver and placental ALPs to hydrophilic dimers. The ALPCA will not, however, release ALP from intact cells which is consistent with the glycosyl-phosphatidylinositol phospholipase from human plasma (22). Further studies with other glycosyl-phosphatidylinositol anchored proteins will be necessary to establish a preferred substrate.

The ALPCA shows several similarities to the glycosyl-phosphatidylinositol-specific phospholipase D from human plasma (Chapter 2, Section G) (3, 22). The M_r, the inhibition by 1,10-phenanthroline, the pH profile, and substrate specificity are consistent between the two enzymes. The M_r of 120 000 compares favorably to the M_r of 110 000 obtained by Davitz et al (22) for the plasma glycosyl-phosphatdiylinositol phospholipase D but differs significantly from the M_r of 500 000 obtained by Low and Prasad (3), although this was possibly due to the formation of multimers or association with other proteins or lipids. The ALPCA does differ from the glycosyl-phosphatidylinositol-specific phospholipase D in its heat stability. ALPCA required 60

min at 70°C for 50% denaturation and was unaffected by 56°C heat whereas the glycosyl-phosphatidylinositol-specific phospholipase D lost 42% of its activity after 30 min at 56°C (Table III-2). However, heat lability of enzymes can be matrix dependent and this could explain the difference. In general, however, our experiments suggest an identity between the phospholipase D found in high concentration in plasma and the integral plasma membrane protein found in human liver reported here.

The presence of equal amounts of ALPCA in whole liver and liver plasma membranes suggests that ALCPA is not concentrated in the plasma membrane. However, there may be several enzymes in whole liver or plasma membranes capable of hydrolyzing the anchor of ALP making this finding difficult to interpret. The increased concentration of ALPCA in serum may indicate that this is the medium in which ALPCA is physiologically active. Alternatively, plasma may be the waste site to allow tissues to remove ALPCA (23).

One proposed function of the glycosyl-phosphatidylinositol anchor is to allow for the selective release of the protein by anchor specific phospholipases (Chapter 2, Section C) (1, 24, 25). The presence of a phospholipase in the plasma membrane may provide a mechanism to release these proteins under

certain stimuli. It may be significant that intact plasma membranes do not convert exogenously added hydrophobic ALP to a hydrophilic form, and similarly Triton-solubilized ALPCA will not cleave ALP from intact membranes. Thus, the significance of the membranous nature of ALPCA and of ALP as a potential substrate is uncertain. Nevertheless, release of glycosyl-phosphatidylinositol anchored proteins by PI-PLC or PI-PLD results in production of diacylglycerol or phosphatidic acid respectively, both of which are potential second messengers (1).

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CHAPTER FOUR

ALKALINE PHOSPHATASE CONVERTING ACTIVITY CLEAVES THE HYDROPHOBIC ANCHOR OFVARIANT SURFACE GLYCOPROTEIN

A. INTRODUCTION

The membrane form of variant surface glycoprotein (mfVSG) can be converted to a water soluble form by bacterial PI-PLC, an endogenous glycosyl-phosphatidylinositol-specific phospholipase C, or a glycosyl-phosphatidylinositol phospholipase D from mammalian plasma (Chapter 2, Sections E and F) (1, 2, 3, 4, 5). Trypanosomes can be cultured in vitro with (9,19-^{3}H)myristic acid resulting in the production of biosynthetically labelled mfVSG (6). The protein can then be isolated and purified providing a sensitive mechanism for assaying the conversion of mfVSG to soluble VSG (1, 7).

This conversion also provided a method with which to determine if ALPCA was capable of removing the hydrophobic anchor from a glycosyl-phosphatidylinositol anchored protein other than

ALP. If ALPCA cleaves the anchor from VSG, the lipid products can then be analyzed by thin layer chromatography to determine the bond specificity of VSG. The assay method for conversion of VSG would also allow for inhibitors of ALPCA to be tested which could not be tested when ALP was the substrate due to inhibition of the catalytic activity of the latter.

B. MATERIALS AND METHODS

1. Materials

Leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), phosphomolybdic acid, 2-(N-morpholino)ethanesulfonic acid (MES), and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Calcium chloride, magnesium chloride, zinc chloride, ScintiVerse II, 1-pentanol, and chloroform were from Fisher Scientific Company, Fair Lawn, NJ, USA. 1-Butanol, methanol, hydrochloric acid, mercuric sulphate, and pyridine were from BDH Inc., Darmstadt, FRG. Triton X-100 was purchased form Boehringer Mannheim, Dorval, Quebec, Canada. HP-K high performance thin layer chromatography plates were from Whatman Inc.,

Clifton, NJ, USA. VSG from trypanosomes that was labelled with (^{3}H)myristate was a generous gift from Dr. Martin Low.

2. ALPCA Cleavage of the Hydrophobic Anchor of VSG

The ability of ALPCA to cleave the hydrophobic anchor from VSG was tested using the method described by Low and Prasad (2). The reaction mixture at 30°C consisted of 32 µl (0.015 mg protein) of Triton-solubilized ALPCA (Chapter 3, Section B-4) and 56 µl of VSG (2000 cpm, 2 µg) diluted in 50 mmol/l MES, pH 6.0 in the presence of 50 µmol/l leupeptin, 250 µmol/l pepstatin A, and 0.1% (w/v) PMSF. The bond specificity of ALPCA was determined by analyzing, after a 4 h incubation, the lipid hydrolysis products of the (^{3}H)myristate-labelled glycosyl-phosphatidylinositol anchor of VSG by thin layer chromatography as described by Low and Prasad (2). PI-PLC from Bacillus cereus and the phospholipase D from human serum were used as phospholipase C and D controls, respectively.

3. The Effect of Activators and Inhibitors on ALPCA Cleavage of VSG

The effect of 1,10-phenanthroline on the ability of ALPCA to remove the VSG anchor was tested by incubating 32 µl of Triton-solubilized ALPCA with 100 µmol/l

1,10-phenanthroline (9 µl of 1 mmol/l) for 5 min at 30°C and then adding 56 µl of VSG (2000 cpm) and incubating for 4 h at 30°C. Controls contained no 1,10-phenanthroline. The ability of zinc chloride to restore ALPCA following 1,10-phenanthroline inhibition was tested by adding $ZnCl_2$ to 105 µmol/l after the incubation with 1,10-phenanthroline. EGTA was tested for its ability to inhibit ALPCA by incubating 32 µl of ALPCA with 2 mmol/l EGTA (9 µl of 20 mmol/l) for 5 min at 30°C followed by the addition of VSG as above and incubation at 30°C for 4 h. Controls contained no EGTA. The ability of calcium chloride, magnesium chloride, and zinc chloride to recover ALPCA following EGTA inhibition was evaluated by adding the ions to 2.1 mmol/l after the 5 min incubation with EGTA. These samples were then mixed gently for 5 min at 30°C followed by the addition of VSG and a 4 h incubation at 30°C. Suitable controls contained either no additions or they contained EGTA and then no further additions. Mercuric sulphate was tested for its ability to inhibit ALPCA by incubating 32 µl of ALPCA with 2 mmol/l mercuric sulphate for 5 min at 30°C followed by the addition of 56 µl of VSG (2000 cpm) and incubation at 30°C for 4 h. Controls contained no additions.

4. The Effect of pH on ALPCA Cleavage of VSG

Triton-solubilized ALPCA was obtained as described in Chapter 3, Section B-4, except that the buffer was 10 mmol/l MES, pH 6.0 rather than 50 mmol/l MES, pH 6.0. The supernatant, containing ALPCA, was adjusted to the appropriate pH values by adding 100 mmol/l MES or 100 mmol/l MOPS at the appropriate pH values. These samples (32 µl) were then mixed with 56 µl of (^{3}H)myristate-labelled VSG (2000 cpm) at the same pH values and incubated at 30°C for 4 h.

C. RESULTS AND DISCUSSION

ALPCA was capable of cleaving the hydrophobic anchor of VSG, the reaction being linear for cleavage of 25% of the substrate (Fig. IV-1). The lipid products of hydrolysis catalyzed by serum phopholipase D or by ALPCA of mfVSG comigrated with dimyrsitoyl-phosphatidic acid while the lipid product of PI-PLC hydrolysis co-migrated with diacylglycerol on thin layer chromatography. This is shown for the latter two enzymes in Plate IV-1. These results taken with previous findings that ALPCA does not hydrolyze non-glycan phospholipids, such as phosphatidylinositol and p-nitrophenylphosphorylcholine (Chapter 3, Section C-7), provide good evidence that ALPCA is a glycosyl-phosphatidylinositol phospholipase D.

ALPCA cleavage of the VSG anchor was completely

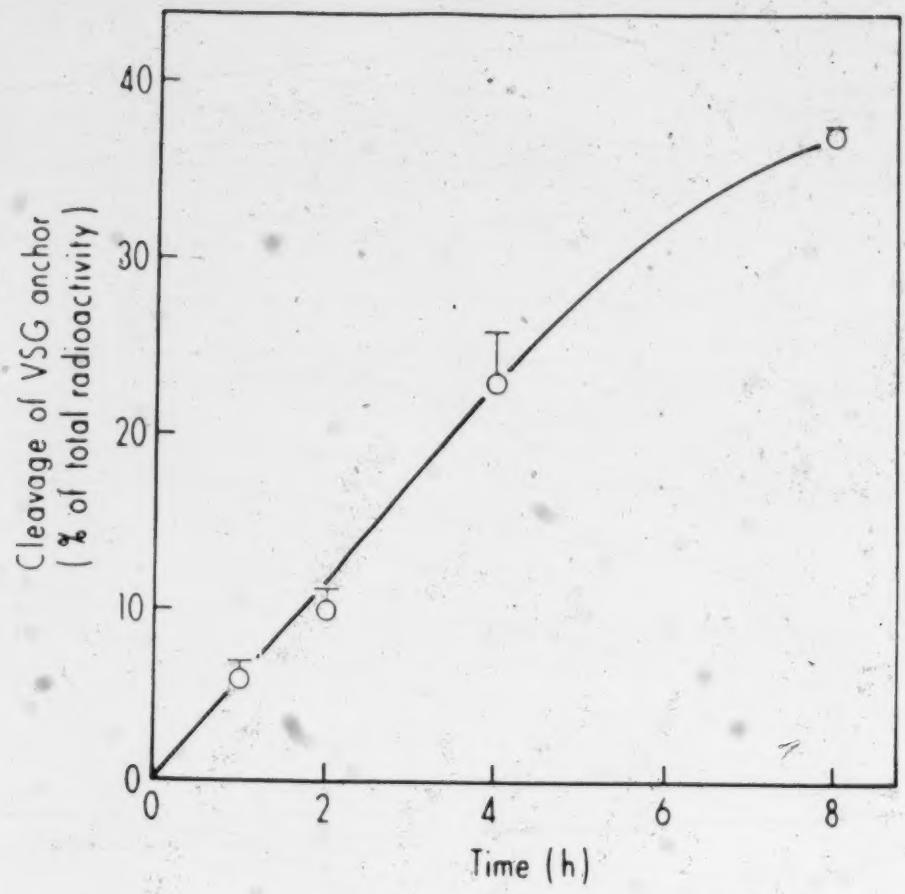

FIGURE IV-1 Time Course for ALPCA Using (^{3}H)Myristate-Labelled VSG as a Substrate. Conversion was at pH 6.0. The bars show 1 SD.

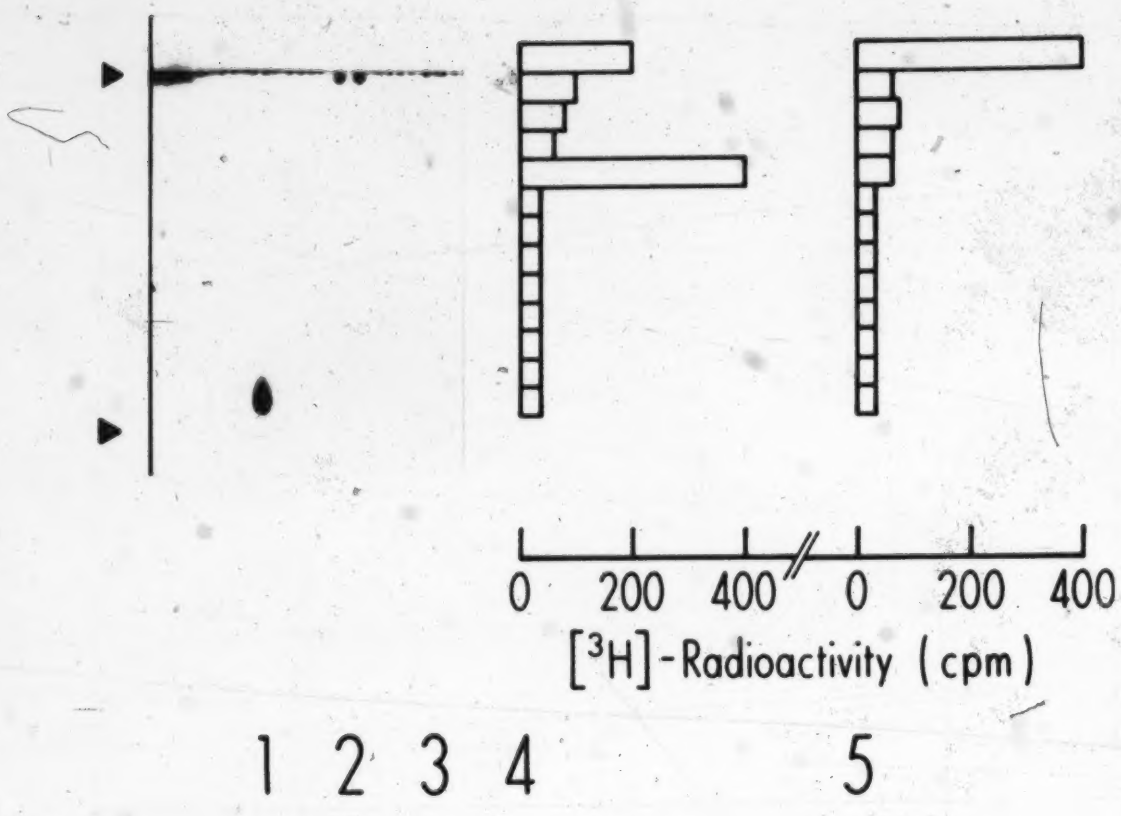

PLATE IV-1 Thin Layer Chromatography of Lipid Products of Phospholipase Hydrolysis. Lane 1, phosphatidylinositol standard; lane 2, diacylglycerol standard; lane 3, phosphatidic acid standard; lane 4, bar graph showing the distribution of (^{3}H)radioactivity following treatment of mfVSG with ALPCA; lane 5 bar graph showing distribution of (^{3}H)radioactivity following treatment of mfVSG with Bacillus cereus PI-PLC.

inhibited by 1,10-phenanthroline at 100 µmol/l, but this activity could be recovered upon addition of excess zinc chloride (Table IV-1). EGTA at 2 mmol/l resulted in approximately 50% inhibition of ALPCA which was completely recoverable with calcium chloride, but only 90% with zinc chloride, and not at all with magnesium chloride (Table IV-1). These ions were ineffective in activating ALPCA in the absence of any inhibition suggesting that zinc and calcium are essential for ALPCA activity. Mercuric sulphate resulted in complete inhibition of ALPCA implicating a thiol group for an active enyzme. Butanol and pentanol both activated the ALPCA cleavage of VSG approximately 2x (Table IV-1), a result consistent with the activation seen when ALP is used as a substrate (Chapter 3, Section C-4). ALPCA cleavage of VSG exhibited a pH optimum at pH 6.0 which is consistent with the pH optimum when ALP is the substrate (Fig. IV-2) (Chapter 3, Section C-6). These data strongly suggest an identity between ALPCA and the phospholipase D of human plasma (2, 3). Thus, this enzyme exists in both soluble and membrane bound forms.

TABLE IV-1 THE EFFECT OF ADDITIONS ON THE ABILITY OF ALPCA TO REMOVE THE MEMBRANE ANCHOR OF VSG*

Condition	Percent Removal of the VSG Anchor (1 SD)
Control	100 (4.4)
1,10-phenanthroline (100 μmol/l)	7 (1.7)
1,10-phenanthroline (100 μmol/l) + $ZnCl_2$ (105 μmol/l)	90 (4.2)
EGTA (2.0 mmol/l)	51 (3.1)
EGTA (2.0 mmol/l) + $CaCl_2$ (2.1 mmol/l)	109 (7.4)
EGTA (2.0 mmol/l) + $ZnCl_2$ (2.1 mmol/l)	90 (7.0)
EGTA (2.0 mmol/l) + $MgCl_2$ (2.1 mmol/l)	49 (14.1)
Mercuric sulphate (2.0 mmol/l)	8 (6.6)
Butanol (1.0 mol/l)	173 (18.9)
Pentanol (375 mmol/l)	205 (13.8)

*The substrate was purified (^3H)Myristate-labelled membrane form VSG in 50 mmol/l MES, pH 6.0.

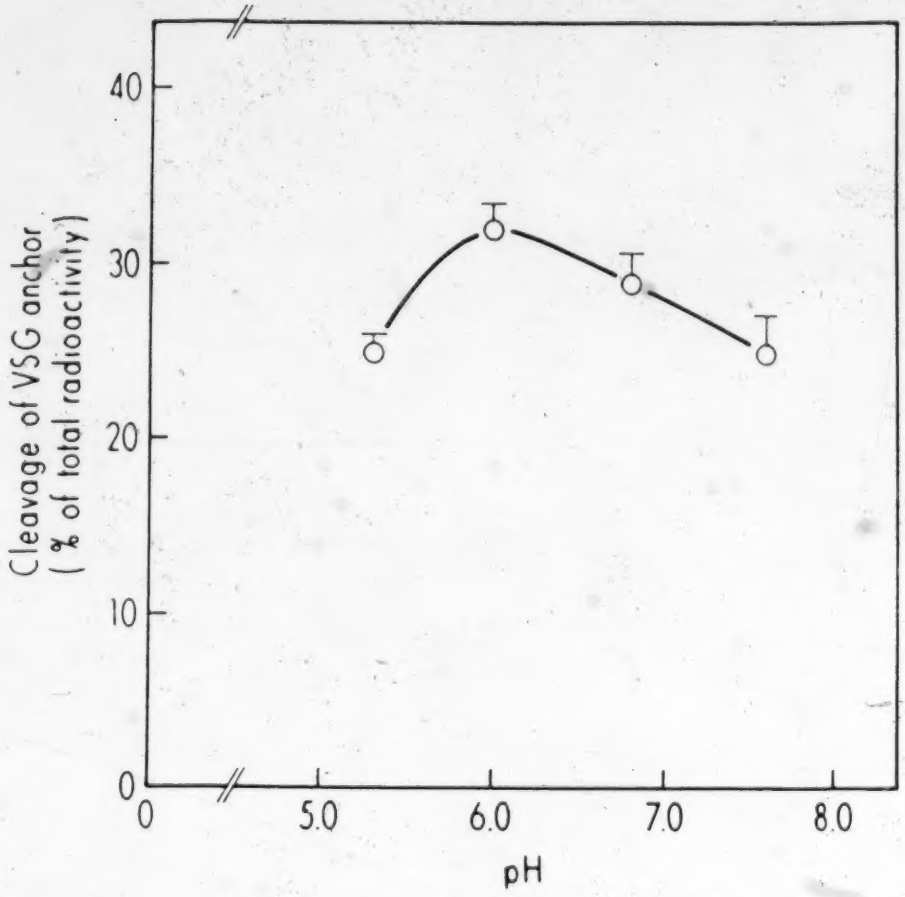

FIGURE IV-2 The Effect of pH on ALPCA Using VSG as a Substrate.

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CHAPTER FIVE

GENERAL CONCLUSIONS

A method was developed for the assay of an enzyme from human liver that removed the membrane phosphatidylinositol anchor from human liver alkaline phosphatase, and thereby converted it from a hydrophobic to a hydrophilic form. The enzyme, referred to as alkaline phosphatase converting activity (ALPCA), was partially purified and characterized such that a tentative identification could be made. Utilization of variant surface glycoprotein (VSG) as substrate in a different assay protocol allowed a confirmatory identification.

ALPCA was determined to be an integral membrane protein due to the requirement of detergents for solubilization from liver plasma membranes. The bond specificity was determined to be that of a phosphlipase D by electrophoresis of the protein products of ALPCA hydrolysis of placental ALP and by thin layer chromatography of the lipid products of ALPCA hydrolysis of VSG. These results combined with the substrate specificity indicate that ALPCA is a glycosyl-phosphatidylinositol phospholipase D.

ALPCA was: most active at acidic pH, stimulated by hydrophobic alcohols, relatively heat stable, and inhibited by 1,10-phenanthroline, EGTA, and mercuric sulfate. These results confirm an identity between the glycosyl-phosphatidylinositol phospholipase D in liver plasma membranes and the one in mammalian plasma.

ALPCA was determined to be approximately 10x higher in serum than in whole liver or liver plasma membranes. The fact that the enzyme activity in plasma membranes and in serum requires some membrane disruption to cleave glycosyl-phosphatidylinositol anchored proteins from intact cells or membranes probably reflects on its physiological significance. More work will be needed to determine if there is a specific stimulus, such as a G-protein, that is necessary for the enzyme to remove proteins from the surface of cells.

This work has shown that phospholipases with glycosyl-phosphatidylinositol sepcificity exist in both soluble and membrane-bound forms. It remains to be ascertained whether both enzyme forms have a physiological role.

APPENDIX A

PURIFICATION OF PI-PLC FROM BACILLUS CEREUS

A. INTRODUCTION

Release of membrane proteins by bacterial PI-PLC enzymes has been the major criteria for determining if a protein is anchored by G-PI (1). Bacteria produce a number of different phospholipases with different substrate specificities and thus it is necessary to obtain a highly purified form of PI-PLC (2). Enzymes with specificity for phosphatidylinositol tend to show the highest degree of specificity (3).

The original procedure we used (4) was modified by Volwerk et al (5). This improved procedure was implemented with a different strain of the organism and the revised methodology is described here.

B. MATERIALS AND METHODS

1. Materials

Bacillus cereus (ATCC 11778) was obtained from the Provincial Laboratory, University of Alberta Hospital, Edmonton, AB, Canada. Ammonium sulfate and sodium chloride were from BDH Inc., Darmstadt, FRG.

L-3-Phosphatidyl(2-^{3}H)inositol was purchased from Amersham Canada Limited, Oakville, ON, Canada. Tris, p-nitrophenyl phosphate, and p-nitrophenylphosphorylcholine were purchased from Sigma Chemical Company, St. Louis, MO, USA. Sephadex G-100 and phenyl-Sepharose CL-4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. The buffer 2-(ethylamino)ethanol was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI, USA. Acrylamide, bis-acrylamide, sucrose, TEMED, ammonium persulfate, and Bio-Rad Silver Stain Kit were purchased from Bio-Rad Laboratories, Richmond, CA, USA. BCA Protein Assay Reagent was from Pierce, Rockford, IL, USA. DEAE-cellulose was purchased from Whatman, Inc., Clifton, NJ, USA. Ethylene glycol, ScintiVerse II, and magnesium chloride were from Fisher Scientific Company, Fair Lawn, NJ, USA. Spectra/por dialysis tubing was from Spectrum Medical Industries Inc., Los Angeles, CA, USA.

2. Purification Procedure

Growth Conditions for Bacillus cereus

The medium contained 40 g Bacto-peptone, 40 g yeast extract, 20 g sodium chloride, 1 g disodium phosphate, and water up to 4 l (5). The pH was adjusted to 7.0 with 1 mol/l sodium hydroxide. The stock culture of B. cereus was stored in transport medium (30 g tryptone soya broth,

5 g glucose, 20 g skim milk powder, 40 ml glycerol, and 1 l of deionized water) at -70°C. A blood agar plate was streaked with the organism and grown overnight at 37°C. A culture was prepared by innoculating 100 ml of media with B. cereus from the blood agar plate and growing for 12 h at 37°C. This culture (50 ml) was then added to two 2 l bottles of media. These were incubated at 37°C in a shaking waterbath for 5 h with air bubbling into the media. The supernatant was recovered by centrifugation for 30 min at 13 000 x g in a Sorvall RC-5 Centrifuge.

Ammonium Sulfate Precipitation

Solid ammonium sulfate was added slowly to 90% saturation (576 g/l) and the solution was stirred for 2 h at 4°C. The solution was then centrifuged for 30 min at 13 000 x g and the dark-brown precipitate was dissolved in 20 mmol/l Tris, pH 8.5 and dialyzed against three changes of the same buffer at 4°C.

DEAE-Cellulose Column

The dialysate was applied to a DEAE-cellulose column (30 ml) equilibrated in 20 mmol/l Tris, pH 8.5. The column was washed with 2-3 column volumes of the same buffer and PI-PLC was eluted with a linear gradient (2 x 300 ml) of 0-0.4 mol/l NaCl in 20 mmol/l Tris, pH 8.5.

Fractions were assayed as described below (Section 3) and those containing PI-PLC activity were concentrated to less than 10 ml using an Amicon ultrafiltration cell with a PM-10 membrane.

Phenyl-Sepharose CL-4B Column

The concentrate from the DEAE column was applied to a phenyl-Sepharose CL-4B column (10 ml) equilibrated in 20 mmol/l Tris, pH 7.5. The column was washed with 2-3 column volumes of the same buffer and the PI-PLC was eluted with 50% ethylene glycol in 20 mmol/l Tris, pH 7.5. The fractions containing PI-PLC were concentrated to 2 ml using an Amicon ultrafiltration cell with a PM-10 membrane.

Sephadex G-100 Column

The concentrate from the phenyl-Sepharose column was applied to a Sephadex G-100 column equilibrated with 20 mmol/l Tris, pH 7.5 and eluted with the same buffer. Fractions containing PI-PLC were pooled, concentrated to 5 ml and stored at -70°C.

3. Enzyme Assays

Plasma Membranes as Substrate

Plasma membranes prepared from healthy human liver and stored at -20°C were resuspended in 50 mmol/l Tris, pH 7.6 (6). Samples (10 µl) containing PI-PLC were added to 50 µl of resuspended plasma membranes and incubated at 30°C for 30 min. The alkaline phosphatase was measured, before and after centrifugation at 14 000 x g for 5 min, to determine the percentage released from the membrane. ALP activity was assayed as described in Chapter 4, Section B-2.

L-3-Phosphatidyl(2-^{3}H)inositol As Substrate

Column fractions (5 µl) were added to 120 µl of L-3-phosphatidyl(2-^{3}H)inositol (4000 cpm) and the solution was incubated at 30°C for 30 min. The mixture was extracted with 250 µl of chloroform/methanol/HCl (100:100:0.6) and 250 µl of 1 mol/l hydrochloric acid. The aqueous layer (250 µl) was added to 4 ml of liquid scintillation fluid (ScintiVerse II) and counted.

p-Nitrophenylphosphorylcholine As Substrate

Column fractions (50 µl) were added to 1 ml of 5 mmol/l p-nitrophenyphosphorylcholine in 50 mmol/l Tris, pH 7.6. This mixture was incubated at 30°C for 2 h. The reaction was stopped by addition of 250 µl of

1 mol/l sodium hydroxide and the absorbance was read at 404 nm on a Perkin-Elmer Lambda 3A UV/VIS Spectrophotometer.

Protein Determinations

Column eluates were monitored at 280 nm and protein was quantitated using the Pierce BCA Protein Assay.

4. Gradient Gel Electrophoresis

PI-PLC (100 µl) before and after treatment with a Sephadex G-100 column was applied to a polyacrylamide slab gradient gel (4-22% polyacrylamide) and electrophoresed for 18 h at 150 V in 0.09 mol/l Tris, 0.08 mol/l boric acid, pH 8.4 (7). The gel was stained for protein using the Bio-Rad Silver Stain Kit.

C. RESULTS

1. DEAE-Cellulose Column

The DEAE-cellulose column resulted in one large peak when the protein concentration was monitored at 280 nm. Two peaks of activity were found when the fractions were assayed with phosphatidylcholine, phosphatidylinositol, or membrane-bound ALP (Fig. APP A-1). The narrow peak showed an enzyme activity with specificity for phosphatidylcholine whereas the large broad peak showed specificity for phosphatidylinositol and ALP.

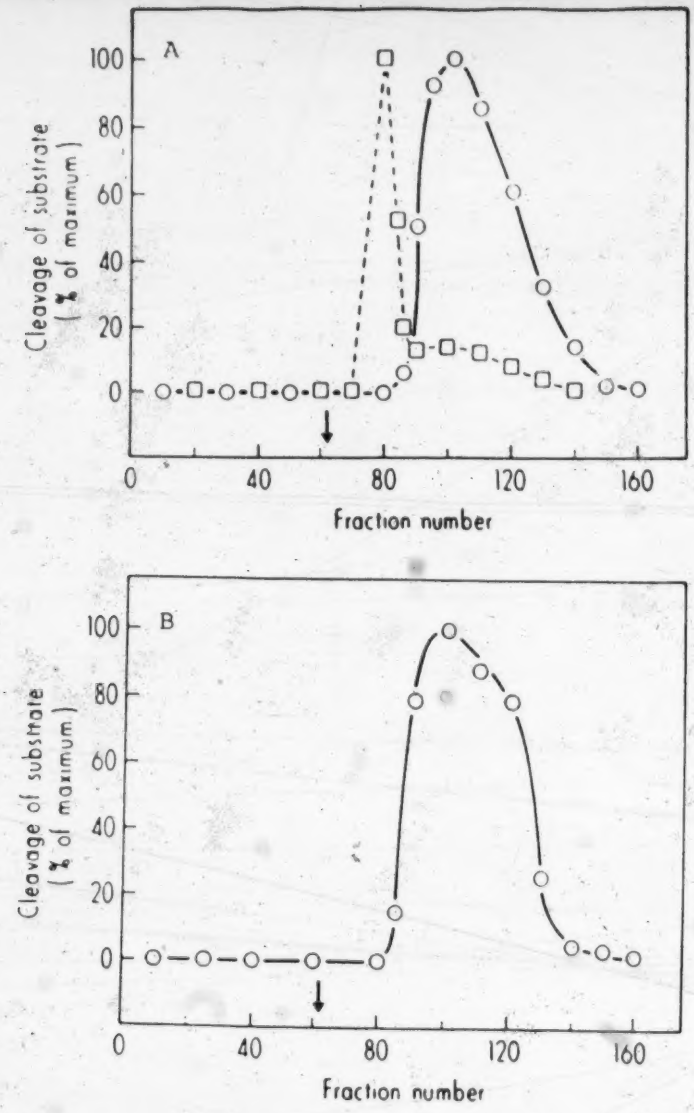

FIGURE APP A-1 DEAE-Cellulose Column. A: Analysis of column fractions using p-nitrophenylphosphorylcholine (□) and membrane ALP (○) as substrate. B: Analysis of column fractions using L-3-phosphatidyl(2-^{3}H)inositol as substrate. The arrow indicates initiation of the sodium chloride gradient.

2. Phenyl-Sepharose CL-4B Column

The phenyl-Sepharose column resulted in two peaks of enzyme activity with phosphatidylinositol as the substrate and two peaks with membrane-bound ALP as the substrate (Fig. APP A-2). None of the fractions exhibited activity towards phosphatidylcholine . The void volume contained PI-PLC which did not adsorb to the column whereas a second peak of PI-PLC activity required ethylene glycol to be eluted from the column. This second peak was the PI-PLC activity that was continued through the purification.

3. Sephadex G-100 Column

The Sephadex G-100 column resulted in one broad peak when analyzed by membrane ALP (Fig. APP A-3). None of the fractions exhibited activity towards phosphatidylcholine. Blue dextran and pyridoxal phosphate were applied as molecular weight standards.

4. Gradient Gel Electrophoresis

Treatment of the PI-PLC with the Sephadex G-100 column resulted in the removal of numerous protein bands (Plate APP A-1). The sample that was applied to the gel filtration column contained one band at a M_r of 27 000 which is probably PI-PLC.

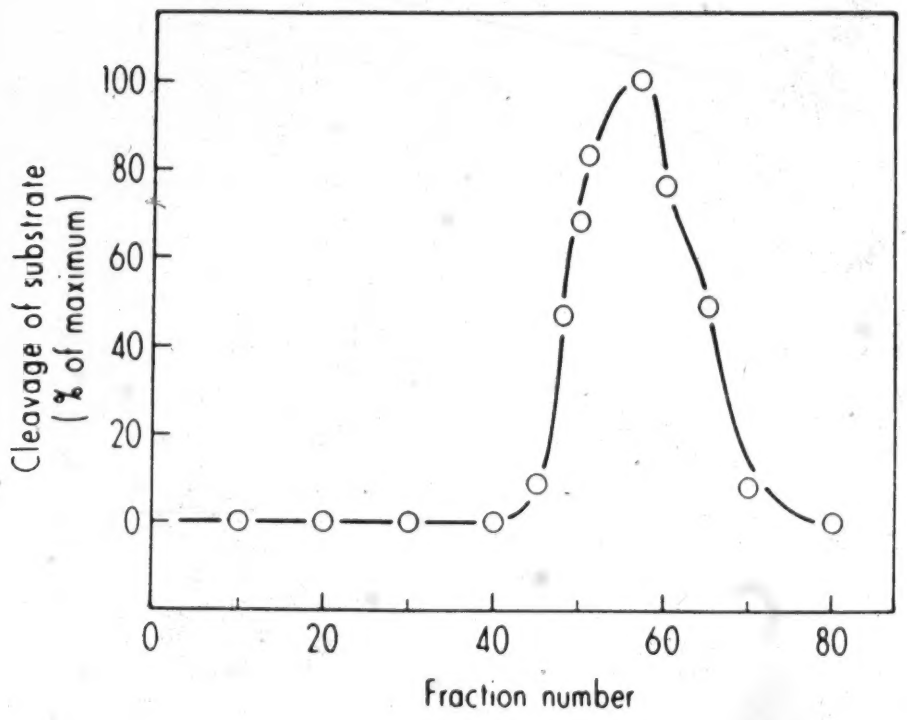

FIGURE APP A-2 Phenyl-Sepharose CL-4B Column. Analysis of column fractions using membrane ALP (○) or L-3-phosphatidyl(2-^{3}H)inositol (□) as substrate. The arrow indicates application of ethylene glycol.

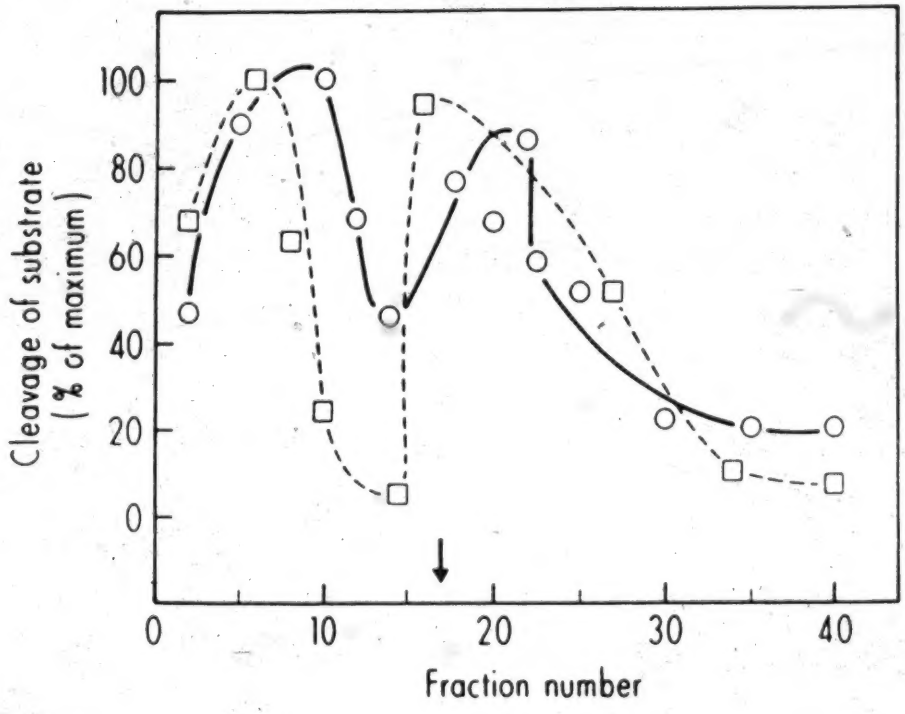

FIGURE APP A-3 Sephadex G-100 Column. Analysis of fractions was with membrane ALP.

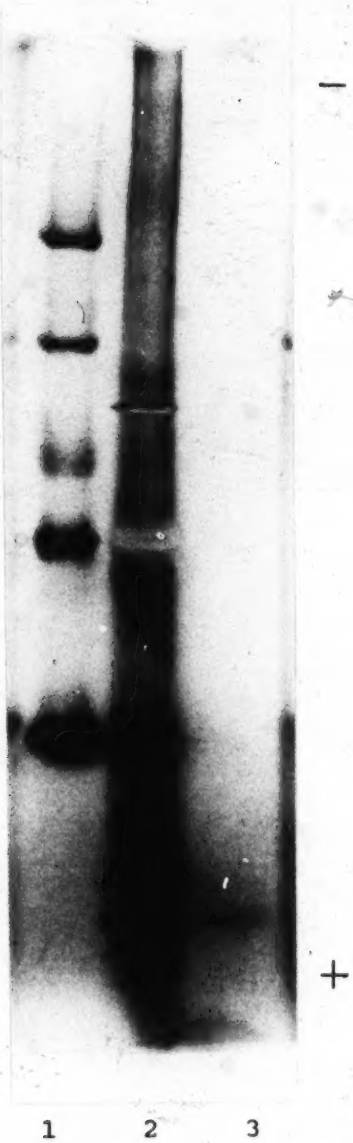

PLATE APP A-1 Gradient Gel Electropherogram of Bacillus cereus PI-PLC. Lane 1, molecular weight standards; lane 2, PI-PLC before Sephadex G-100 column; lane 3, PI-PLC after Sephadex G-100 column.

D. DISCUSSION

The PI-PLC obtained from this new purification procedure was much purer than the PI-PLC obtained from the original procedure. The addition of the gel filtration column, which was suggested by other purification protocols, resulted in the removal of a number of proteins (Plate APP A-1) (9). The $\underline{M}_r$ of 25 500 and 26 500 obtained by gel filtration chromatography and gradient gel electrophoresis, respectively compare favorably to the $\underline{M}_r$ of 29 000 obtained by Ikezawa et al (10).

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APPENDIX B

PUBLICATIONS ARISING FROM THIS THESIS

1. Hamilton, B.A., McPhee, J.L., Hawrylak, K., and Stinson, R.A. Alkaline phosphatase releasing activity in human tissues. Clin. Chim. Acta. 186:249-254, 1990 .